Fatty Acid Metabolism in Cyanobacteria

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

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Abstract

With crude oil demand rising and supplies being depleted, alternative energy, specifically biofuels, are of intense scientific interest. Current plant crop based biofuels suffer from several problems, most importantly the use of land needed for food. Cyanobacteria offer a solution to this problem as they do not compete with land for food and produce hydrocarbons that can be used as biofuels. Upon examination of metabolic pathways competing with hydrocarbon synthesis, it appeared that cyanobacteria lacked the major fatty acid degradative metabolic pathway β-oxidation, generally thought to be a universally occurring pathway. Lack of this pathway in cyanobacteria was confirmed by employing a range of analytical techniques. Bioinformatic analysis suggested that potential enzymes with β-oxidation activity were involved in other metabolic pathways. A sensitive assay was set up to detect acyl-CoAs, the substrates of β-oxidation, using liquid chromatography triple quadrupole mass spectrometry. None could be detected in cyanobacteria. No enzymatic activity from the rate-limiting acyl-CoA dehydrogenase/oxidase could be detected in cyanobacterial extracts. It was found that radiolabeled fatty acids fed to cyanobacteria were utilised for lipid membranes as opposed to being converted to CO_2 by respiration or into other compounds by the TCA cycle. An element of the β-oxidation pathway, *E. coli* acyl-CoA synthetase was ectopically expressed in a strain of cyanobacteria and implications of the introduction of acyl-CoA synthesis were assessed. Finally, the regulation of the fatty acid biosynthetic pathway was investigated. It was determined that under conditions of excess fatty acid, the transcription of acetyl-CoA carboxylase and enoyl-ACP reductase was repressed and acyl-ACP synthetase involved in fatty acid recycling was induced. These results were discussed in relation to fatty acid oxidation and hydrocarbon biosynthesis in other organisms.

Contents

List of Tables

List of Figures

List of abbreviations

ACN	acetonitrile
ACP	acyl carrier protein
ANOVA	analysis of variance
ASW	artificial sea water
BB	BioBrick
BCCP	biotin carboxyl carrier protein
BG-11	BG-11 cyanobacterial growth medium
BLAST	Basic Local Alignment Search Tool
bp	base pairs of DNA
BSA	bovine serum albumin
BSTFA	*N,O*¬-*bis*(trimethylsilyl)trifluoroacetamide
cDNA	complementary DNA
CID	collision-induced dissociation
CoA	coenzyme A
Da	daltons
DCM	dichloromethane

DGDG	digalactosyldiacylglycerol
DMSO	dimethyl sulfoxide
DPM	disintegrations per minute
dsDNA	double stranded DNA
DTT	dithiothreitol
EDC	1-ethyl-3,3-dimethylaminopropylcarbodiimide
EDTA	ethylenediaminetetraacetic acid disodium salt
EIA	Energy Information Administration
ESI	electrospray ionisation
FAD	flavine adenine dinucleotide
FadD	acyl-CoA synthetase
$FADH_2$	reduced flavine adenine dinucleotide
FAME	fatty acid methyl ester
FAR	fatty acyl reductase
g	relative centrifugal force
GCMS	gas chromatography mass spectrometry
gDNA	genomic DNA
HICR111A	*Acaryochloris* strain HICR111A
HPLC	high performance liquid chromatography
IAA	iodoacetamide
IncP	incompatibility group P
INT	iodonitrotetrazolium
KAS	ketoacyl-ACP synthase
Kb	Kilobase pairs
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LB	Luria Bertani bacterial culture broth
LC-MS	liquid chromatography mass spectrometry
LC-QToF-MS	liquid chromatography quadrupole time of flight mass spectrometry

LC-QQQ-MS	triple quadrupole liquid chromatography mass spectrometry
m/z	mass to charge ratio
MBIC 11017	*Acaryochloris marina* strain MBIC 11017
MeOH	methanol
MGDG	monogalactosyldiacylglycerol
MRM	multiple reaction monitor
mRNA	messenger RNA
MS	mass spectrometer
MS/MS	tandem mass spectrometer
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NH4Ac	ammonium acetate
NIST	National Institute of Standards and Technology
nt	nucleotides
OD	optical density
OECD	Organisation for Economic Co-operation and Development
OPEC	Organisation of the Petroleum Exporting Countries
ORF	open reading frame
PC	phophatidylcholine
PCC 6714	*Synechocystis* strain PCC 6714
PCC 6803	*Synechocystis* strain PCC 6803
PCC 7120	*Nostoc* strain PCC 7120
PCC 73102	*Nostoc punctiforme* strain PCC 73102
PCC 7421	*Gloeobacter violaceus* strain PCC 7421
PCC 7425	*Cyanothece* strain PCC 7425
PCC 7937	*Anabaena variabilis* strain PCC 7937
PCR	polymerase chain reaction
PE	phosphoethanolamine

Pfam	Protein Family Database
PFD	photon flux density
PG	phosphoglycerol
PHA	polyhydroxyalkanoate
PMS	phenazine methosulfate
ppm	parts per million
PrbcL	promoter region of RuBisCO large subunit gene
QToF	quadrupole time-of-flight mass spectrometer
RFP	red fluorescent protein
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase polymerase chain reaction
RuBisCO	ribulose bisphosphate carboxylase oxygenase
SEM	standard error of mean
SOB	super optimal broth
SPE	solid phase extraction
SQDG	sulfoquinovosyldiaylglycerol
SRM	single reaction monitor
TAE	tris-acetate-EDTA buffer
TAG	triacylglycerol
TE	tris-EDTA buffer
TIC	total ion chromatogram
TLC	thin layer chromatography
U	enzymatic units – amount of enzyme required to catalyse 1 µmol of substrate per min at 25 °C.

1. Introduction

1.1 The need for sustainable biofuels

Since the onset of the industrial revolution in the late 18th century, humans have been reliant on fossil fuels for industry and transportation. Coupled with the dramatic increase seen in world population during this time period, and the industrialisation of many countries that were previously undeveloped, there has been a depletion of these fossil fuels, of particular interest is that of crude oil. Crude oil was formed under conditions of low heat and pressure acting upon biological matter over a time period of millions of years (Berner, 2003), and as such, it is essentially non-renewable.

Crude oil is primarily used for the production of liquid fuels for transportation, a sector that currently relies almost entirely on this natural resource, in addition heating, road surfacing and lubricants rely on crude oil (U.S Energy Information Administration). As of 2010, crude oil accounted for 48 % of the world's global energy source, with coal second at 29 %, nuclear at 11 % and renewables accounting for 10 % (EIA Annual Energy Outlook, 2012). Overall oil demand is predicted to fall in the period of 2010 to 2035 in OECD countries by 4.2 million barrels per day (Mb/d), due to improvements in efficiency of transport, industrial processes and subsidies granted to renewables by the respective countries' governments. On the other hand, in developing countries that are experiencing industrialzation, oil demand is set to rise by 26 Mb/d. An overall global increase in oil demand of 6.1 Mb/d (OPEC World Oil Outlook, 2011).

Another reason that global demand is set to increase is due to increasing global population. Although the rate of population increase is falling, from a high of 35 births per 1000 of population in 1960, to 20 births per 1000 of population in 2010, predicted to be 10 births per 1000 of population in 2035 (Population Division of the Department of Economic and Social Affairs of the United Nations Secretariat, 2010), the overall global population is predicted to increase from 7 billion today to 8.5 billion by 2035.

The increase in demand for crude oil has already seen prices rise from $75 /b in 2010 up to $120 /b in 2011. This increase in demand has been shown to be a cause of conflicts such as those seen in Iraq, Kuwait and Libya. Whilst crude oil will not for a very long time physically run out, the cost of extracting the remaining oil is likely to be too high to be able to compete with other energy sources, thus no longer making it a viable option as an energy source.

In addition to the issues caused by dwindling oil supplies as described above, oil is a fossil fuel and so contributes to anthropogenic CO_2 emissions. Increasing CO_2 levels have been shown to have a direct correlation with increasing global temperatures, with CO_2 levels between 200 - 290 ppm before the onset of the industrial revolution dating back 420,000 years, inferred by analysis of gas bubbles in ice cores (Petit et al. 1999), having risen to 391.07 ppm as of September 2012 according to the Mauna Loa Earth System Research Laboratory, Hawaii (http://co2now.org) (Keeling et al. 1976; Thoning et al. 1989). These values are directly correlated with a rise in global average temperatures over the same time period, having been observed independently by many scientific groups (Baringer et al. 2010).

Effects of anthropogenic climate change include global sea level rise, loss of habitat, increased poverty, extinction of species and reduced biodiversity. Perhaps the most well documented effect of climate change is the melting of polar ice caps, but also the less well documented warming of the oceans, has also contributed to rising sea levels due to the thermal expansion of water (Wigley and Raper, 1987), resulting in habitat loss and destruction of ecosystems. Other effects of climate change include habitat loss caused by temperatures increasing faster than residing organisms (in particular plants) can adapt to them, resulting in lower productivity, which consequently will affect crop yields and therefore food security. This is especially important in developing nations that lie in the tropics, where productivity has been predicted to fall by up to 40 % (Battisti and Naylor, 2008). Habitat loss may be caused by desertification, although it

is thought that over-cultivation and overgrazing are the primary causes of this rather than climate change (Le Houerau, 1995). At the other end of the scale, there is evidence that increased global warming may result in increased or altered rainfall patterns in specific regions (Wentz et al. 2007), again resulting in flooding and potential loss of habitat. Increased global average temperatures are not the only effect of anthropogenic CO_2 emissions. Oceans have taken up 30 % of all CO_2 released to date (Cai et al. 2010) and as this dissolves in the seawater, it dissociates into carbonate and bicarbonate-containing salts as well as carbonic acid. This has the effect of lowering the oceans pH, which also has a negative effect on marine life (Hoegh-Guldberg et al. 2007). Additionally, the capacity of the oceans to absorb gases with increasing temperature decreases (Duan and Sun, 2003), meaning that coupled with the increase in global temperatures, CO_2 levels would continue to rise to even higher levels. Many groups have attempted to model what the concentration of CO_2 will be, with estimates in the range of 550 ppm to 700 ppm by 2050 and concurrent increases predicted in global average temperatures (Solomon et al. 2007).

Another drawback of crude oil is that reserves are only found in particular regions, resulting in some countries having an excess of oil, such as those in the OPEC group, but at the same time other countries having to import oil to satisfy their energy needs. Thus, crude oil results in many countries having energy dependency on others, which can have a negative effect on these countries' economies and the quality of life of their inhabitants, as well as increasing the possibility of conflicts between these countries as they strive to obtain access to these resources.

One alternative to crude oil-derived products are biofuels. By definition, biofuels are fuels that are derived directly from living matter. This includes solids, such as plant material, agricultural waste and bio-char; liquids such as ethanol, vegetable oil and biodiesel; and gases such as biogas, syngas and biohydrogen (Demirbas, 2009). The biofuel that has been in use the longest by humans for energy generation is plant

matter, in particular wood, being burned for heat for at least the last 230,000 years (James, 1989). In terms of transport, the most common biofuels currently used are ethanol derived from sugarcane in Brazil or corn in the USA and biodiesel derived from the seed oils of crops such as rapeseed, indeed one of the first diesel cycle engines created by Rudolph Diesel in the late 19th century ran on peanut oil (Nitschke and Wilson, 1965), these types of "traditional" biofuel are sometimes termed First Generation biofuels.

The major advantage of biofuels versus crude oil-derived fuels is that they are renewable. As such, it will eventually be cheaper to produce biofuels than extract crude oil for fuel. Biofuels also have the potential to offer energy security for nearly all countries, improving the quality of life for these countries' inhabitants and reducing competition for resources between nations that can lead to international tensions. Some biofuels may result in lower net CO_2 emissions, however biofuels are generally not carbon-neutral (Johnson, 2009).

Crop based biofuels suffer from several other problems; they use up large amounts of arable land (Hill et al. 2006) that could otherwise be used for production of food crops, resulting in increased food prices. They suffer from low yields in terms of starting biomass (Chisti, 2008) and slow growth, especially compared to microorganisms. These fuels also require further processing, for example the raw product that comes from crops of rapeseed for the production of biodiesel are typically triacylglycerides, that need to undergo a transesterification reaction to remove the glycerol group (Huber et al. 2006), not only is this process costly, but it also produces an unwanted by-product in the form of glycerol and requires methanol which is usually derived from natural gas, a non-renewable fossil fuel. As a direct result of seed oil crop based biodiesel, markets are currently experiencing a worldwide glycerol glut (Johnson and Taconi, 2007).

Next-generation or advanced biofuels can refer to biofuels that are typically produced by the conversion of feedstocks into a useable fuel source. Feedstocks may include lignocellulosic biomass or agricultural and municipal waste, which do not directly compete with arable land used in food production, these feedstocks are converted into a useable fuel source either by thermochemical or biochemical processes (Inderwildi and King, 2009). Thermochemical processes include gasification, torrefaction and pyrolysis, and biochemical processes typically employ fermentation by microbes to convert the feedstocks into fuel. One major disadvantage of thermochemical processes are the large amounts of energy input required. Currently a large amount of biofuels research is being carried out on a number of microbial subjects such as bacteria (Kalscheuer, 2006); fungi and in particular yeast (Ratledge and Wynn, 2002) and microalgae (Banerjee et al. 2002). Microalgae perhaps offer the most exciting solution to the question of efficient microbial biofuels, as they have the ability to fix atmospheric carbon dioxide directly into a fuel source, with a reduced requirement for additional costly pre- and post-processing steps.

1.2 The Cyanobacteria

1.2.1 The Cyanobacteria – An overview

The cyanobacteria, formerly known as blue-green algae, are a phylum of Gram-negative bacteria, although they share some features of Gram-positive bacteria (Jugens et al. 1983). Along with plants and algae, cyanobacteria are capable of oxygenic photosynthesis. Green and purple sulfur- and non-sulfur bacteria are also capable of photosynthesis, but can utilize sulfur, hydrogen sulfide or hydrogen and amino- or other organic acids as their electron donors instead of water respectively (Olson, 2006). Cyanobacteria are descendants of the first organisms that performed oxygenic photosynthesis, with cyanobacteria-like fossils having been found in Warrawoona, Australia dating back to 3.5 billion years ago (Schopf, 1993) and are the

organisms that can be credited with changing the earth's atmosphere from that of a CO_2-rich, reducing atmosphere to the oxygen rich one that is present today, with the 'Great Oxidation Event' occurring approximately 2.4 billion years ago (Caitling and Clare, 2005; Buick, 2008).

The endosymbiotic theory states that cyanobacteria are the ancestors of plastids (Margulis, 1970; Bogorad, 1975), found not only as chloroplasts in algae and plants but also in parasitic protists such as *Plasmodium falciparum* and *Toxoplasma gondii,* having lost their photosynthetic function (Lang-Unnasch et al. 1998). The initial hypothesis of endosymbiosis was first postulated in the early 20th century (Martin and Kowallik, 1999). Evidence supporting the endosymbiotic theory includes observations that chloroplasts divide by binary fission in the same way as cyan obacteria (Glynn et al. 2007), have circular chromosomes like bacteria, but are much reduced in size as many of the genes have been transferred to the host nucleus (Timmis et al. 2004) and their ribosomes share the same 70S sedimentation coefficients as bacteria, rather than eukaryotic 80S ribosomes. Additionally the glaucophyte alga *Cyanophora paradoxa* contains a type of primitive chloroplast, termed a cyanelle, which has retained the peptidoglycan layer in its cell wall (Steiner and Loffelhardt, 2002; Pfanzagl et al. 1996) and also the genes for peptidoglycan synthesis are essential for chloroplast division in the moss *Physcomitrella patens* (Machida et al. 2006).

Cyanobacteria, being prokaryotic are classed as unicellular organisms, but some species can also arrange into filamentous forms. They inhabit a wide variety of ecological niches, for example freshwater, saltwater, soil, high and low temperatures, on rocks and in symbiosis with other organisms. Three of the strains studied in laboratory experiments in this thesis were isolated from freshwater environments; *Synechocystis* PCC 6803 (Fig. 1.1, B), was originally isolated from a freshwater lake near Berkeley, CA, USA by Riyo Kunisawa in 1968 (Stanier et al. 1971); *Nostoc sp.* PCC 7120 (Fig. 1.1, C) from an unknown freshwater source in the USA (Adolph and

Haselkorn, 1971) and *Anabaena variabilis* PCC 7937 (Fig. 1.1, D), isolated from a freshwater sewage pond in Mississippi, USA circa 1964 (Wolk, 1964). Species of cyanobacteria from genera such as *Prochlorococcus* and *Acaryochloris* are found in saltwater environments. *Acaryochloris* HICR111A (Fig. 1.1, A) that was also studied in laboratory experiments for this thesis, was isolated from Heron Island, Queensland, Australia in the Great Barrier Reef by Mohr et al. circa 2005 and was found growing as a biofilm over a dead coral skeleton. Other strains live in soil, such as *Microcoleus* (Booth, 1941) some in extreme environments, ranging from the hot, arid high-deserts of the Colorado Plateau (Garcia-Pichel et al. 2001) to the polar deserts of the Antarctic (Pandey et al. 2004). *Gloeobacter violeaus* PCC 7421 was isolated from moistened calcerous rocks as part of a biofilm, by Rosmarie Rippka near Lucerne in Switzerland in 1972 (Rippka et al. 1974). Many cyanobacteria form symbiotic relationships with other organisms, such as *Nostoc punctiforme* PCC 73102 that associates with species of the genus of higher plants *Gunnera* by entering the plant through glands found at the base of the leaf stalk (Bergman et al. 1992). Other examples of symbiosis include *Nostoc* species in lichens (Honegger, 1991), *Oscillatoria* that lives on the hair of Sloths (Wujek and Lincoln, 1988), giving the animals a green hue that provides camouflage, and in marine poriferans (sponges) (Webster and Taylor, 2012) and ascidians (sea-squirts) (Lopez-Lengentil et al. 2011).

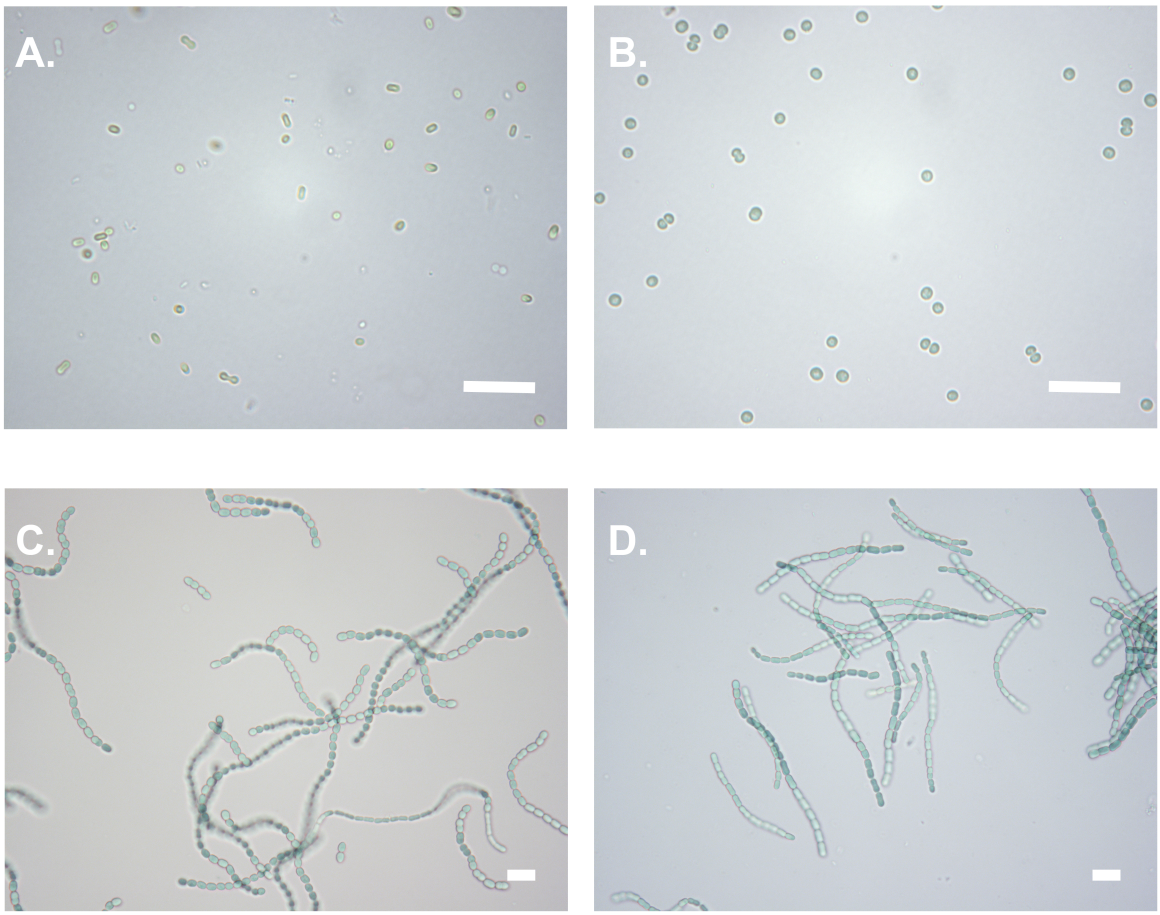

Figure 1.1. Bright field images of cyanobacteria that were studied. A. *Acaryochloris* HICR111A; B. *Synechocystis* PCC 6803; C. *Nostoc* PCC 7120; D. *Anabaena variabilis* PCC 7937. Scale bar represents 20 μm.

Another area in which the cyanobacteria exhibit great diversity is in metabolism. In addition to CO_2 fixation by photosynthesis, some strains of cyanobacteria possess the required characteristics to fix atmospheric nitrogen into its bioavailable, reduced form of ammonia. Some colonial filamentous strains, such as PCC 7120 and PCC 7937 develop specialized cells within their filaments under conditions of nitrogen starvation called heterocysts (Adams, 2000). Some other filamentous and unicellular cyanobacterial strains such as those in the genus *Oscillatoria* and *Cyanothece* do not form heterocysts, yet can still fix N_2 under aerobic conditions (Bergman et al. 1997). Cyanobacteria have evolved mechanisms to protect the, highly conserved, nitrogenase enzyme complex that is irreversibly inhibited by molecular oxygen (Postgate, 1998).

Yet another example highlighting the metabolic diversity of the cyanobacteria is a group of compounds for which cyanobacteria are infamous, toxins. The first published report on cyanobacterial toxins was described in the late 19th century at the estuary of the Murray River where it flows into Lake Alexandrina as a “thick scum like green oil paint, some two to six inches thick, as thick and as pasty as porridge.” the organism responsible putatively identified as *Nodularia spumigena,* being observed to kill cattle within a time period of one to sixteen hours from being ingested (Francis, 1878). The main groups of cyanobacterial toxins are the nonribosomal cyclic peptides, alkaloids and polyketides. Nonribosomal cylic peptides include the seven-amino acid microcystins and the five-amino acid nodularins, which are hepatotoxins that affect the liver by interfering with formation of microfilaments in the hepatocytes’ cytoskeleton, resulting in the affected cells breaking contact with other hepatocytes and capillaries (Carmichael, 1994), only liver cells generally are susceptible to these toxins as they possess the necessary ATP-dependent transport proteins that are able to import these compounds into the cell (Runnegar et al. 1981). They have also been shown to have tumour promoting activity by showing inhibition of protein phosphatases 1 and 2A (Fujiki and Sugmuna, 1999). Alkaloids are comprised of anatoxins, saxitoxins, cylinderospermopsins and lyngbyatoxins, named after their respective genera from which they were isolated. Anatoxins mimic the neurotransmitter acetylcholine that is released at the synapses of neurons and muscle cells and stimulates muscle cells to contract, except that unlike acetylcholine, anatoxins cannot be degraded by acetylcholinesterase, resulting in overstimulation of the muscle cell, which leads to cramping, followed by fatigue, paralysis and death if respiratory muscles are affected (Carmichael et al. 1975). Saxitoxins are neurotoxins like anatoxins, except they act on the axon of the neuron instead of the synapse, preventing the generation of an electrical impulse by blocking the inward flow of sodium ions, causing paralysis (Mahmood and Carmichael, 1986). Cylindrospermopsin acts mainly on the liver (Hawkins et al. 1985) and inhibits protein synthesis by covalently modifiying DNA and

RNA (Shaw et al. 2000). Lyngbyatoxins are potent blistering agents and irritants, causing a condition known as seaweed dermatitis (Cardellina et al. 1979) as well as having tumour-promoting effects (Fujiki et al. 1981). The polyketide aplysiatoxin also has irritant and carcinogenic effects (Arcoleo and Weinstein, 1985). Cyanobacterial lipopolysaccharides (LPS) have also been attributed as potential irritants, although whether it is LPS that are the causative agents remains under debate (Stewart et al. 2006). Cyanobacteria strains that are known to produce toxins include those from the genera *Microcystis, Anabaena, Oscillatoria, Nostoc, Cylinderospermopsis, Lyngbya, Moorea, Nodularia* and *Aphanizomenon.*

In addition to photoautotrophy, many cyanobacteria can exhibit a degree of heterotrophy, being able to utilize exogenous carbon sources for growth, such as glucose in PCC 6803 (Smith, 1982), *Prochlorococcus* SS120 (Gomez-Baena et al. 2008), *Calothrix* PCC 7601 (Lebedeva et al. 2005) and *Cyanothece* ATCC 51142 (Schneegurt et al. 1997). These strains require short exposure to low intensity light every day in order to remain viable, yet this light is not enough to maintain the cell's growth alone - this feature is termed light-activated-heterotrophic-growth (Anderson and McIntosh, 1991). Some strains such as *Synechocystis* PCC 6714 and *A. variabilis* strains ATCC 29133 and ATCC 29413 are capable of fully heterotrophic growth (Ludwig et al. 2008; Mannan and Pakrasi, 1993), with the two *A. variabilis* strains also being capable of utilizing fructose, ATCC 29413 exclusively using this sugar (Haury and Spiller, 1981).

In addition to growth on sugars, many cyanobacteria are also able to take up and use other compounds such as acetate (Ihlenteldt and Gibson, 1977), amino acids (Montesinos et al. 1996) and even components of complex hydrocarbon mixtures that are found in crude oil spillages (Raghukumar et al. 2001; Ibraheem, 2010; Cerniglia et al. 1980). Note however that no reports of fully-heterotrophic- or light-activated-heterotrophic-growth on these types of compound have been documented.

1.2.2 Hydrocarbon biosynthesis in cyanobacteria

Conversely to what was mentioned above about cyanobacteria being able to degrade hydrocarbons, many strains of cyanobacteria are also able to synthesise these compounds. The first report in 1969 documented the presence of hydrocarbons between 15 and 18 carbons (C15-C18) in chain length, as well as significant amounts of 7- and 8-methylheptadecane in *N. muscorum* after feeding ^{14}C-labeled fatty acid (Han et al. 1969), this publication co-authored by Melvin Calvin of Calvin-Benson cycle fame. A year later, 2 further reports documenting the synthesis of alkanes in cyanobacteria were published; one showing that *A. varibilis* was able to incorporate radioactivity from the feeding of tritiated methionine into alkanes (Fehler and Light, 1970) and another report detailing the hydrocarbon compositions of 11 cyanobacterial strains, showing that the marine strains tested accumulate only C19 mono- and di-enoic alkenes (Winters et al. 1969). Utilising stable isotope labeling with ^{13}C- and ^{2}H-acetate feeding coupled to NMR, McInnes et al. (1980) showed that stearic acid is the likely precursor of heptadecane in the biosynthetic pathway of hydrocarbons in *Anacystis nidulans*. Further publications from 1982 and 2002 have also documented the occurrence of hydrocarbons in *Spirulina* using liquid chromatography (Rezanka et al. 1982) and gas-chromatography-mass-spectrometry (GCMS) (Dembitsky and Srebnik, 2002). It was only recently that the biosynthetic pathway of alkane production in cyanobacteria was elucidated (Schirmer et al. 2010). This was achieved using subtractive genome analysis, as one of the eleven cyanobacterial strains that they analysed, *Synechococcus* PCC 7002, lacked alkanes. Two candidate genes from the other strains stood out, one having predicted short chain reductase activity and the other having similarity to ribonucleotide reductases, which were subsequently identified and characterized as an acyl-ACP reductase and aldehyde decarbonylase respectively. PCC 7002, whilst lacking alkanes, did have C19 mono- and di-enoic alkenes present, as documented by Winters et al. (1969). Mendez-Perez et al. (2011) identified the biosynthetic pathway for these compounds, catalysed by a large

mulitdomain enzyme with homology to type I polyketide synthases.

1.2.3 The commercial importance of cyanobacteria

The fact that many cyanobacteria can produce hydrocarbons, and that some even degrade them as described above, makes cyanobacteria commercially valuable. Other commercial uses for cyanobacteria include for the food industry, both animals and humans, providing sources of protein, polyunsaturated fatty acids and pigments (Spolaore et al. 2006), in particular phycocyanins that are found in cyanobacteria (Viskari and Coyler, 2003); in medicine and pharmacology, with some products from cyanobacteria having antitumour (Tan, 2007) and antibiotic properties (Borowitzka, 1995) and even in the cosmetic industry (Stolz and Obermayer, 2005).

In terms of candidates for the production of biofuels, cyanobacteria have several advantages over eukaryotic species of microalgae; They are generally simpler in metabolic and genomic respects, for example they have no cell compartmentation, and genomes that are not bound to histones. In theory this makes it easier to characterise and manipulate metabolic and gene expression pathways. Unlike most algae, cyanobacteria additionally possess a CO_2 concentrating mechanism (Aizawa and Myachi, 1986). This enhances photosynthetic CO_2 fixation by concentrating CO_2 around the carbon-fixing enzyme, ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) to 1000 fold the concentration normally found within the cell. They do this in structures called carboxysomes that contains RuBisCO and carbonic anhydrase that converts HCO_3^- to CO_2, making it available to RuBisCO, as well as having active HCO_3^- transporters in their plasma membrane (Badger and Price, 2002) to increase overall photosynthetic efficiency. This biomolecular simplicity coupled with highly efficient photosynthesis results in higher growth rates in cyanobacteria than eukaryotic microalgae. For example, one of the fastest growing eukaryotic model algae *Chlamdomonas reinhardtii* has a mean doubling time of 5 hours under optimal

conditions (Lee and Fiehn, 2008), whereas the cyanobacterial species *Synechococcus* PCC 7002 has a mean doubling time of 2.6 hours (Bryant et al. 2012).

1.3 Carbon flux throughout metabolism

Current yields of hydrocarbons from cyanobacteria are low. For example PCC 6803 has a heptadecane yield of 2 μg per mg of dry cell mass (see appendix, section 6.4), taking into account the density of heptadecane of 0.777 g l^{-1} and assuming 100 mg cyanobacteria are present in 1 ml of culture, approximately 3891 litres of cyanobacteria would be needed to produce 1 litre of heptadecane. Therefore commercial production of heptadecane from cyanobacteria is currently not viable.

There are a number of ways in which the productivity of hydrocarbon production from cyanobacteria could be improved. One way is by increasing the level of primary production by increasing the efficiency of photosynthesis. This has been studied extensively in the field of plant and crop science, with efforts to engineer the C_4 photosynthetic pathway into C_3 plants to eliminate photorespiration (Zhu et al. 2010; Sage and Zhu, 2011). However as stated in 1.2.3, cyanobacteria already have a CO_2 concentrating mechanism, but that is not to say that efficiency could not be improved upon in other areas of photosynthesis such as light harvesting. In illuminated cultures the light intensity decreases pseudo-exponentially with increasing depth into the culture (Nakada et al. 1995), meaning that cells deep within the culture receive little to no light, while cells on the surface receive more light than they can use to drive photosynthesis, resulting in photodamage (Melis, 2009). A solution to this problem is to reduce the number of light-harvesting complexes associated with photosystems, meaning that more light can pass through the cells at the edges of the culture and can reach the cells in the centre. This has been demonstrated in a number of algae, cyanobacteria and other non-oxygenic photosynthetic bacteria. By knocking out the light-harvesting antenna assembly protein encoding gene *tla2* in *C. reinhardtii*, Kirst et al. (2012) were able to reduce the size of the antennae by 65 %, resulting in improved solar energy

conversion in bright light and mass culture conditions. In the photosynthetic, hydrogen producing, purple non-sulfur bacteria *Rhodobacter capsulatus*, a regulatory protein, PufQ was overexpressed under the cytochrome C oxidase subunit promoter *Pcbb3*, the gene of which was knocked out (Ma et al. 2012). PufQ is thought to repress bacteriochlorophyll synthesis. It was found that the resulting mutant had an overall lower absorption of light between 300 and 900 nm and that hydrogen production increased by 27 % over wild type. Nakajima and Veda (1999) knocked out phycocyanin production in the cyanobacterial strain PCC 6714 and reported 50 % higher productivity at high light intensities compared to wild type.

Another way in which yield of hydrocarbons could be improved is by considering what happens to the carbon once it is fixed: its metabolic fate within the cell. Modifying the flux of carbon throughout metabolism so that carbon is diverted away from non-essential pathways and sinks and into the desired end product. For the purpose of hydrocarbon production in cyanobacteria this would mean diverting as much carbon as possible into the fatty acid biosynthetic pathways, as acyl-ACPs are precursors to these compounds, and diverting carbon away from other pathways. Figure 1.2 gives a simplified overview of the major metabolic pathways in cyanobacteria that could be modified in order to improve yield. The pathways that contribute carbon towards hydrocarbon biosynthesis are 1. the photosynthetic carbon fixation pathway, the Calvin cycle, resulting in glycerate-3-phosphate (G3P); 2. the conversion of this compound to the central metabolite acetyl-coenzyme A (acetyl-CoA) via glycolysis; 3. the synthesis of acyl-ACP precursors of hydrocarbons in the fatty acid biosynthetic pathway; 4. the conversion of acyl-ACP thioesters to hydrocarbons by acyl-ACP reductase and aldehyde decarbonylase. All other metabolic pathways are therefore in competition for carbon from CO_2 fixation with the 3 latter pathways mentioned above. However it would not be possible to knockout or knockdown all of these pathways, because some are essential to the organisms survival and/or are required for the desired pathways to function, amino acid synthesis for example is necessary for the

proteins that catalyse all the reactions required for synthesis of hydrocarbons and is also required for the cells survival, so it would not be feasible to downregulate this metabolic pathway.

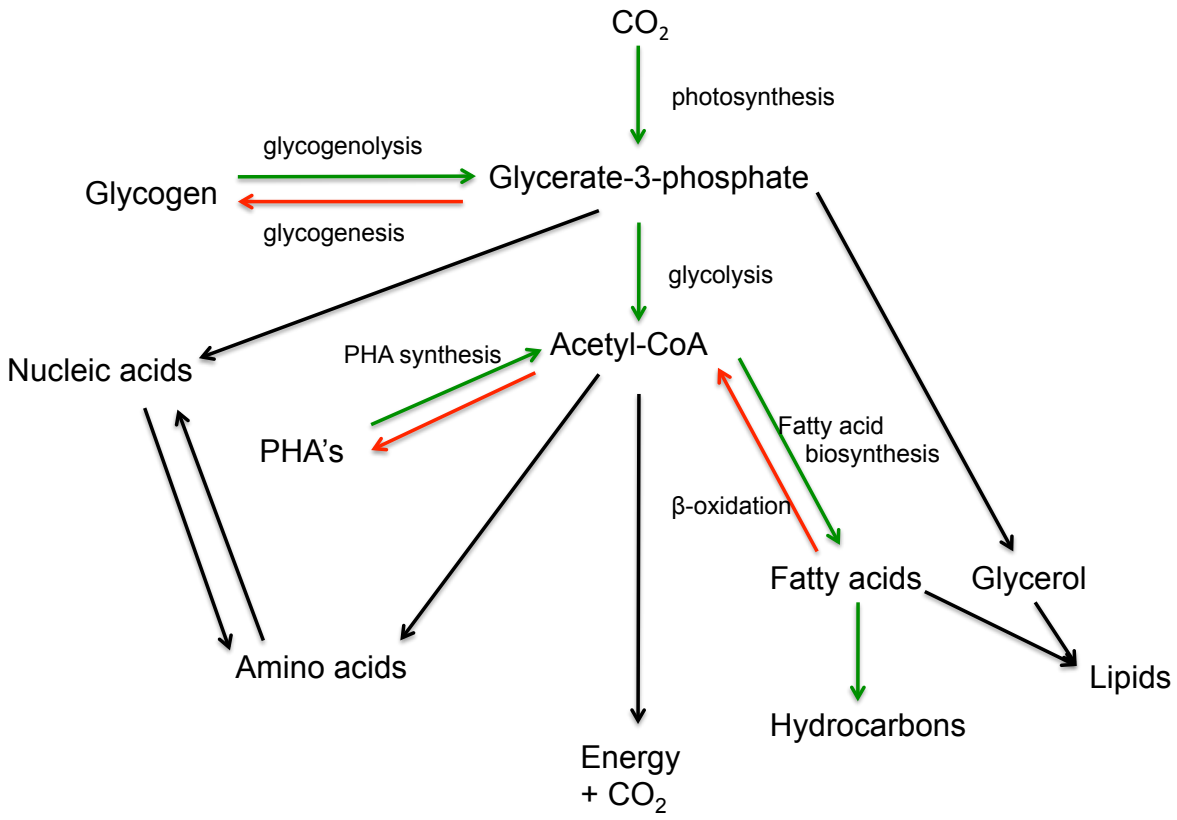

Figure 1.2 A simplified overview of metabolism and the carbon flux with regards to hydrocarbon biosynthesis. Green arrows show essential and non-essential pathways that divert carbon flow towards hydrocarbons; red arrows show pathways that could possibly be downregulated with minimal negative pleiotropic effects on the organism; black arrows show other pathways that are probably essential to the organisms' survival.

Two pathways that could be possible candidates for downregulation in order to improve yield of hydrocarbons are the glycogen biosynthetic pathway, glycogenesis and the fatty acid degradative pathway β-oxidation.

1.4 Glycogen metabolism

Unlike higher plants that use starch, cyanobacteria store excess sugars in the form of glycogen (Smith, 1982) as do animals and yeast. Glycogen is a polysaccharide consisting of α-1,4-linked glucose monomers that have α-1,6-links at the branching points. Molecular weights are between 10^7 and 10^8 Da. In bacteria glycogen accumulates under conditions of excess carbon and limiting concentrations of nutrients (Eydallin et al. 2007). The mechanisms of glycogenesis and glycogenolysis are highly conserved in the bacteria (Ballicora et al. 2003) and the regulation of these metabolic pathways is highly interconnected and complex (Wilson et al. 2010), as such a simple overview is given here.

G3P from carbon fixation is converted into glycogen via a series of 8 reactions (Fig. 1.3). The first 5 reactions result in the formation of α-D-glucose-6-phosphate, which acts as a substrate for 3 metabolic pathways: 1. synthesis of β-D-glucose, 2. the pentose phosphate pathway, or 3. glycogen synthesis. The pentose phosphate pathway is required for the biosynthesis of ribose sugars that are essential components of many important molecules such as DNA, RNA and ATP. Therefore the gene encoding the enzyme that is responsible for the synthesis of α-D-glucose-6-phosphate, glucose-6-phosphate isomerase (*pgi* gene in cyanobacterial strains PCC 6803 and PCC7120) would not be a suitable candidate for deletion, as this would likely result in cell death as not only glycogen synthesis would be inhibited, but also synthesis of nucleotides, which in turn would inhibit DNA, RNA, protein synthesis and ATP synthesis altogether.

The following reaction, where α-D-glucose-6-phosphate is converted to α-D-glucose-1-phosphate by phosphoglucomutase (*pgm* gene in PCC 6803), looks like a key reaction in the synthesis of glycogen. This gene has not been characterised in strain PCC 7120, though it likely exists (Whitehouse et al. 1998). If this gene were to be knocked out, then α-D-glucose-1-phosphate could not be synthesised and the enzymes further up

the glycogen synthesis pathway would have no substrate with which to synthesise glycogen. α-D-glucose-1-phosphate is not used in any other pathways that are critical to the survival of the organism, so knock out of this gene should not be lethal – a *pgm* null mutant generated in a nitrogen fixing soil bacteria species *Mesorhizobium loti* contained an altered form of lipopolysaccharide, lacked exopolysaccharide, beta cyclic glucan, and glycogen. They did not investigate lipid overproduction, but crucially it confirmed that the cells survive and glycogen synthesis was halted (Lepek et al. 2002).

Another important enzyme of glycogen synthesis is encoded by *glgA* (also annotated as *agp*), glycogen synthase (also known as ADP-glucose pyrophosphorylase). This enzyme catalyses the final step of glycogen biosynthesis. This gene has been knocked out in PCC 6803, and it was found that no glycogen was synthesized, but large amounts of sucrose were detected (Miao et al. 2003a), so α-D-glucose-1-phosphate was being converted into sucrose instead of glycogen by the enzymes UTP-glucose-1-phosphate uridylyltransferase and sucrose synthase (*spsA*) (Curatti et al. 1998) via a UDP-glucose intermediate. Furthermore, in a later investigation, they found that the *agp* mutant had 38% less photosynthetic capacity compared to wild type PCC 6803 under high light conditions, which indicates that there is a minimum requirement for glycogen synthesis for normal growth of this species (Miao et al. 2003b).

Increasing the rate of glycogenolysis i.e. the breakdown of glycogen, would serve to deplete the cells of glycogen entirely, so reducing the impact of this carbon sink even further and enhancing carbon flow into fatty acids. The enzyme responsible for initiating glycogenolysis is glycogen phosphorylase, encoded by *glgP* genes. Two of these genes are found in *Synchococcus* PCC 7002 (Jacobsen et al. 2011) and in PCC 6803 (Fu and Xu, 2006), where they also found that one *glgP* mutant resulted in a lack of growth at high temperatures and the second *glgP* mutant resulted in the cells being unable to utilise the glycogen under conditions of starvation. Overexpression of the *glgP* genes would result in increased glycogenolysis. It should be noted that if

glycogenolysis was to be increased in this way then deletion of *pgm* would not be feasible, as the enzyme catalyses both forward and backward reactions and so is needed for conversion back the to glycerate-3-phosphate starting point. If both *glgP* was overexpressed and/or *pgm* was deleted, then there would probably be an observed increase in cellular concentrations of α-D-glucose-1-phosphate. Therefore the optimal strategy would be to overexpress *glgP* and delete *glgA*, although *glgP* overexpression may not be necessary if *glgA* is deleted as no glycogen would be synthesised in the first place.

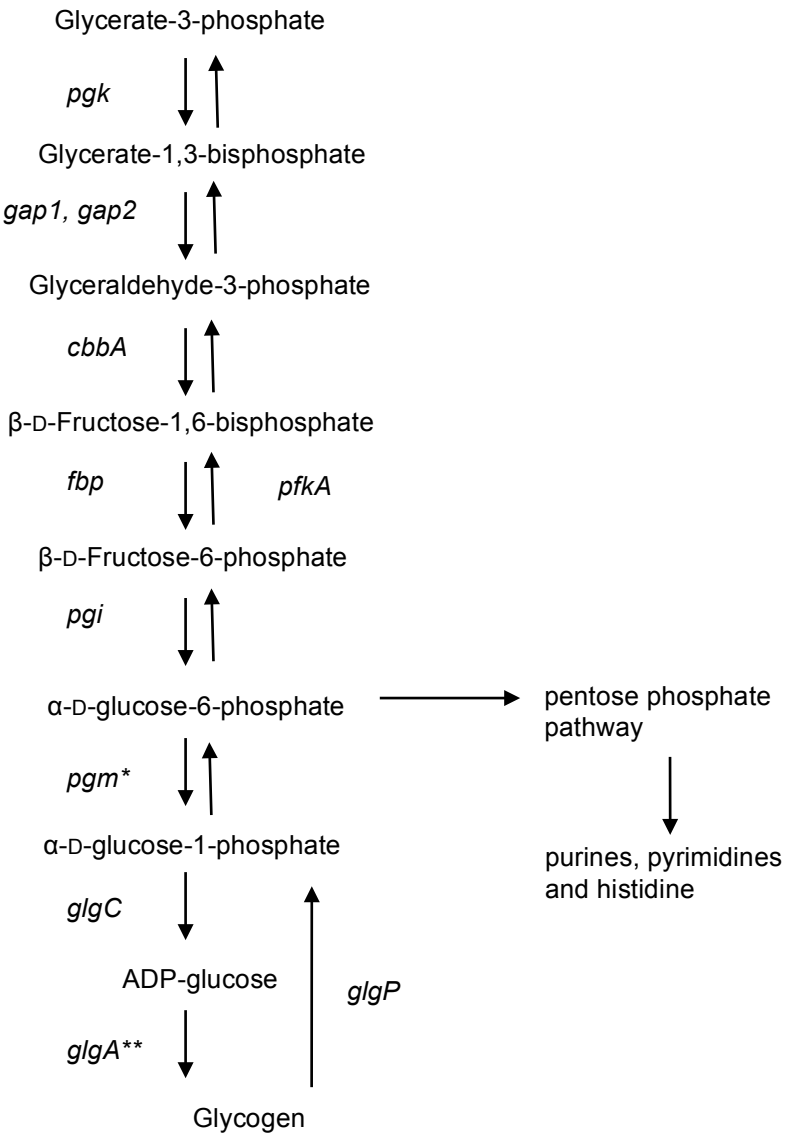

Figure 1.3. Overview of glycogen metabolism from glycerate-3-phosphate in PCC 6803 and PCC 7120. Shown are the main metabolic intermediates, and the encoding genes for enzymes that catalyse steps in the pathway. *- this gene is not characterised in *Anabaena* PCC 7120. **- also annotated as *agp*. Pathway derived from KEGG (Kanehesa and Goto, 2000).

1.5 Fatty acids

Fatty acids and lipids of which fatty acids are constituents are a diverse group of compounds, with regards to both structure and function. They fulfil a variety of roles within the cell, their main functions being energy storage and as components of cell membranes. Other functions include signalling. Unlike proteins, nucleic acids and polysaccharides, they are not polymers, but can still associate with one another through noncovalent interactions.

The general structure of a fatty acid is that of a polar carboxylic acid end attached to a hydrocarbon chain. The carboxylic acid is weakly acidic, with pK_a values of approximately 4.5 to 4.75 and so exist as carboxylate ions at physiological pH. The hydrocarbon chain can be anything from 2 to 80 carbons in chain length, but is more commonly in the region of 10 up to 24 in most organisms; they also usually exist as even number chain lengths due to the nature of fatty acid biosynthesis, but odd chain length fatty acids do exist primarily in bacteria and plants (Rezanka et al. 2009). Acyl chain lengths of fewer than ten carbons are termed short chain fatty acids, carbon chains in the range of 10 to 14 are medium chain fatty acid and over 14 carbons in length are referred to as long chain fatty acids. The polar, hydrophilic carboxylate group and nonpolar, hydrophobic hydrocarbon or acyl chain give this class of compounds an amphipathic nature. The molecule arranges itself in water in such a way that the carboxylate end associates with the water molecules and the acyl chains are repelled away from the water, causing the fatty acids to clump together in micelles or as a monolayer on the water/air interface. This feature gives fatty acids a useful role in forming membranes found in cells, though typically fatty acids are found as part of complex lipids such as glycerophospholipids or glyceroglycolipids.

Due to the presence of the highly reduced acyl chain, fatty acids make very good energy storage molecules, as they will release a high yield of energy upon oxidation. For example the energy released from one mole of palmitic acid releases 129 moles of ATP compared to 38 moles of ATP from 1 mole of glucose oxidised. The energy yield is also greater per carbon oxidised; 129/16 = 8.063 ATP's / carbon for palmitic acid versus 38/6 = 6.34 ATP's / carbon for glucose.

When acting as energy stores, fatty acids are found bound to glycerol within the cell, as triacylglycerides (TAG). These have a very low solubility within the cell and do not tend to form micelles or bilayers like the more polar lipids and so exist in the cell as insoluble fat droplets. TAGs however have not been detected in cyanobacteria (Alvarez and Steinbuchel, 2002), so fatty acids may serve more of a structural rather than energy storage function.

1.6 Fatty acid biosynthesis

There are two different systems by which fatty acids are synthesised; the eukaryotic system and the prokaryotic system. In eukaryotes, fatty acid synthase carries out all of the reactions of fatty acid biosynthesis, having multiple catalytic activites. In prokaryotes these reactions are carried out by separate enzymes. Plants utilise the prokaryotic system of fatty acid biosynthesis, these enzymes are localised in the chloroplast. The reactions catalysed by the enzymes of fatty acid biosynthesis are the same whether they are performed by the eukaryotic type fatty acid synthase complex or by prokaryotic enzymes. The overview of fatty acid biosynthesis outlined here is applicable to prokaryotes, generally using *E. coli* as the model organism as that is where most work has been carried out, unless otherwise stated. An overview of fatty acid biosynthesis is given in Fig 1.4.

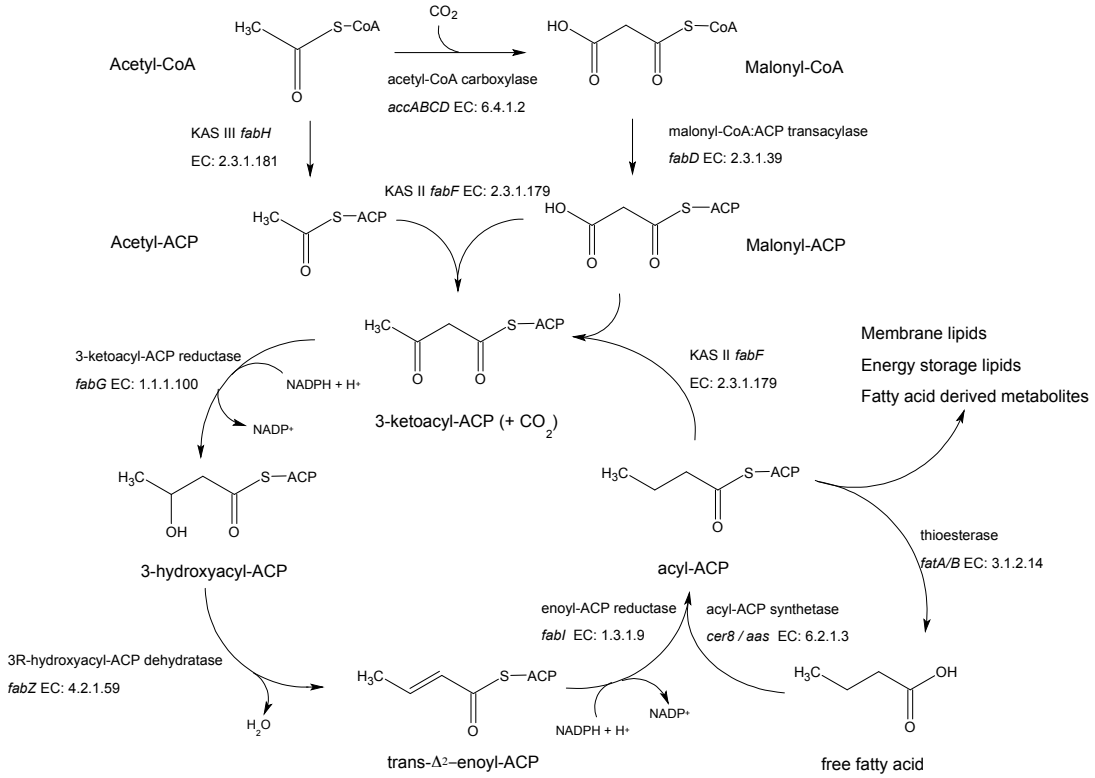

Figure 1.4. Overview of fatty acid biosynthesis. Initiating reaction of acetyl-CoA plus CO_2 to malonyl-CoA is carried out by the acetyl-CoA carboxylase complex. Conversion of acetyl-CoA and malonyl-CoA to their repective ACP thioesters is performed by KAS III and malonyl-CoA:ACP transacylase. These two compounds are subsequently condensed together by KAS II to form a 3-ketoacyl-ACP, plus CO_2. The following series of reductions by 3-ketoacyl-ACP reductase, 3R-hydroxyacyl-ACP reductase produce an acyl-ACP product. This can then be condensed with another molecule of malonyl-ACP and undergo another round of reduction to form an acyl-ACP 2 carbons in length longer. This cycle continues until an acyl-ACP of desired size is achieved, typically 16 or 18 carbons in length, at which point they enter into other biochemical pathways where they act as precursors. In cyanobacteria acyl-ACP synthetase can ligate free fatty acids to ACP thioesters that can enter the cycle of elongation.

1.6.1 Coenzyme A

The starting substrate of fatty acid biosynthesis is acetyl-CoA, which is synthesised from pyruvate in the glycolytic pathway. Coenzyme A itself is synthesised from β-alanine and pantoate in a series of 6 reactions. β-alanine is synthesised from the amino acid aspartate by aspartate-1-decarboxylase (encoded by *panD*) (Cronan, 1980) and pantoate is synthesised from a series of three reactions from the amino acid valine, which involve enzymes from genes *ilvE, panB* and *panE*. β-alanine and pantoate form pantothenate (also known as vitamin B5) via a reaction catalysed by PanC. *PanBCD* are all cotranscribed in an operon in *E. coli* (Merkel and Nichols, 1996).

The next step involves the phosphorylation of pantothenate by pantothenate kinase (*coaA*) gene. This is an important step in the synthesis of coenzyme A as it is a rate-controlling step of CoA synthesis. Pantothenate kinase activity is regulated by feedback inhibition by CoA itself (Vallari et al. 1987). It is not the only rate-limiting step in CoA synthesis though, as a later reaction catalysed by phosphopantethine adenylyltransferase (*coaD*) that converts 4'-phosphopantetheine into dephospho-CoA also has regulatory function (Jackowski and Rock, 1984). Dephospho-CoA is finally phosphorylated into CoA by dephospho-CoA kinase (*coaE*) (Mishra et al. 2001).

1.6.2 Formation of malonyl-CoA by the acetyl-CoA carboxylase complex

The first true step in fatty acid biosynthesis, i.e. the point of no return for acetyl-CoA, is the carboxylation of acetyl-CoA to form malonyl-CoA. In bacteria (including cyanobacteria) and plants this is carried out by a multienzyme complex called acetyl-CoA carboxylase, which is 113 KDa in size in PCC 6803. This is composed of four subunits; biotin carboxyl carrier protein (BCCP) encoded by *accB*, biotin carboxylase (*accC*) and an α- and β subunit of carboxyltransferase encoded by genes *accA* and *accD* respectively. BCCP contains a biotin cofactor, which binds the carboxyl group that is transferred to acetyl-CoA to form malonyl-CoA. The biotin cofactor is ligated to

BCCP by biotin ligase, an enzyme that can repress its own transcription by binding to the operator of the *birA* gene with the aid of the corepressor biotinoyl-AMP, the product of the first half-reaction of ligation in *E. coli* and *Bacillus subtilis* (Cronan, 1989). In *E. coli*, biotin is synthesised via a series of 5 reactions by the *bio* genes (Bower et al. 1996) from pimelate – itself produced from ω-oxidation of fatty acids by ω-oxidase enzymes.

The function of the biotin carboxylase protein is to bind the carboxyl (COO) group from HCO_3^- to the biotin cofactor of BCCP, using an Mn^{2+} cofactor. α-carboxytransferase and β-carboxytransferase form a heterodimer and perform the catalytic function of condensing the carboxyl group onto acetyl-CoA to form malonyl-CoA.

Increasing activity of these genes results in an increased pool size of malonyl-CoA, and so an increase in rate of fatty acid production. Overproduction of fatty acids in *E. coli* has been observed in mutants that overexpress *acc* genes (Lu et al. 2008).

The eukaryotic form of this protein is somewhat different, existing as a single subunit in a dimeric form when inactive, but in the presence of citrate it polymerises to a very large filamentous form between 4 MDa and 8 MDa in size (Lane et al. 1974). Plants contain both the eukaryotic and prokaryotic forms of this enzyme (Alban et al. 1994), the prokaryotic form being found in the plastid.

1.6.3 Acyl carrier protein (ACP)

The malonyl group on malonyl-CoA is transferred to ACP by malonyl-CoA:ACP transacylase encoded by *fabD*. Acetyl-CoA is converted to acetyl-ACP by 3-Ketoacyl-ACP-synthase III (gene *fabH*). These carrier proteins make the malonyl and acetyl groups available to the later enzymes of fatty acid synthesis.

ACP itself is synthesised in an inactive form from the gene *acpP* as apo-ACP. The 4'-phosphopantetheine prosthetic group from CoA is transferred to Ser-36 via a

phosphodiester linkage on apo-ACP by Acyl carrier protein synthase (*acpS* gene) to give the active form of ACP, holo-ACP (Magnuson et al. 1993).

Overproduction of apo-ACP (or a lack of post translational modification by AcpS) inhibits cell growth, by competitively inhibiting the *sn*-glycerol-3-phosphate acyltransferase, resulting in an inability to transfer the completed fatty acid to the *sn*-glycerol-3-phosphate (Keating et al. 1995), resulting in the cell not being able to synthesise glycerophospholipids that are used in cell membranes, and so inhibiting growth.

1.6.4 Fatty acid biosynthesis

Acetyl-ACP and Malonyl-ACP are condensed together by 3-Ketoacyl-ACP-synthase III (KAS III), to form 3-ketobutyryl-ACP and CO_2. The 3-keto group is then reduced by 3-ketoacyl-ACP reductase (encoded by *fabG*) and NADPH as the reducing agent to give 3-hydroxybutyryl-ACP.

The hydroxy group that has been formed on carbon 3 is then dehydrated by 3-hydroxyacyl-ACP dehydratase (*fabZ* gene) to give trans-Δ^2-butenoyl-ACP (and H_2O). In *E. coli*, on the fourth round of this dehydration reaction, another enzyme, β-hydroxydecanoyl-ACP dehydrase (encoded by *fabA*) can perform the dehydration. Under certain conditions, for example lowered temperature, isomerisation of the carbon-carbon double bond between carbons 3 and 4, instead of between carbons 2 and 3, is catalysed by this enzyme, resulting in a cis-conformation double bond. This results in production of an unsaturated fatty acid (Henry and Cronan, 1992). The *fabA* gene is not present in cyanobacteria, and generation of unsaturated fatty acids is performed and regulated by a different class of enzymes encoded by the *des* genes (Wada and Murata, 1990), these are discussed in 1.8.

In saturated fatty acid synthesis, the double bond can then be reduced by 2,3-trans-Δ^2-enoyl-ACP reductase (encoded by *fabI*), with NADPH as the reducing agent to form

butyryl-ACP. The butyryl-ACP produced in the previous reaction is then condensed with another malonyl-ACP molecule to form a 3-ketoacyl-ACP that is 6 carbons in length (3-ketohexanoyl-ACP) and CO_2. This then undergoes the same series of reactions as described from the beginning of this section, increasing the length of the acyl chain by two carbons each time. This step is carried out in cyanobacteria by 3-ketoacyl synthase II (KAS II), which is encoded by *fabF*; however in *E.coli* this can either be carried out by KAS I or II.

In addition to the acyl chain elongating enzymes mentioned above, cyanobacteria posess acyl-ACP synthetase, which ligates free fatty acids to ACP (Kaczmarzyk and Fulda, 2010). This enzyme is encoded by a gene called *aas* and bears significant homology to *Arabidopsis* long chain acyl-CoA synthetase 1, encoded by *cer8* (Lu et al. 2009).

1.6.5 Final steps of fatty acid biosynthesis

The long chain acyl-ACP's produced can undergo a variety of different metabolic fates. They can be; 1. Transferred into complex structural lipids to produce compounds such as glycerophospholipids or glyceroglycolipids that are used in cell membranes and lipoproteins; 2. Transferred into energy storage complex lipids such as triacylglycerides; 3. They can undergo thiolytic cleavage to yield free fatty acids; 4. Enter other biochemical pathways for the synthesis of novel or secondary metabolites - such as hydrocarbons that have been described in cyanobacteria, waxes that are found in plants, or steroids and other hormones.

In phospholipid biosynthesis in *E.coli,* the acyl-ACP is transferred onto the glycerolphosphate backbone by two enzymes: glycerol-3-phosphate-O-acyltransferase, the *plsB* gene product, and 1-acyl-*sn*-glycerol-3-phosphate acyltransferase, which is the *plsC* gene product. This is an area of metabolism that consumes fatty acids, so could possibly be inhibited in order to yield greater quantities of fatty acids, however, inhibition of glycerophospholipids could inhibit cell growth or even prove lethal to the

cells, as they are the main component of cell membranes. Gene knockouts of equivalents in *B. subtilis* resulted in halting of glycerophospholipid production, but a continued high rate of fatty acid production (Paoletti et al. 2007). However, a lack of glycerophospholipid synthesis has previously been proven to inhibit cell growth (Keating et al. 1995). The *plsB* and *plsC* equivalents have not been characterised in cyanobacteria, though a hypothetical protein equivalent to *plsC* is present according to genome annotation.

Production of free fatty acids by thioesterases is not well characterised in cyanobacteria. Some strains appear to have required protein sequences with the required activity according to genomic annotation, but these have not been characterised. The strains that have these sequences are *A. variabilis*, PCC 7120, PCC 73012, *Microcoleus chthonoplastes* PCC 7420, *N. spumigena*, *Cyanothece* strains PCC 7424, ATCC 51142 and CCY 0110, *Oscillatoria* strains and *G. violaceus* PCC 7421. These thioesterases could be involved in the synthesis of non-ribosomal peptides and toxin production.

In *E. coli*, 2 thioesterases have been characterised: one is a multifunctional acyl-CoA thioesterase, protease and lysophospholipase, encoded by *tesA* and known as thioesterase I, the other is encoded by *tesB* and is known as acyl-CoA thioesterase II. Thioesterases from plants such as *Umbellularia californica* have been transformed into bacterial strains (Voelker and Davies, 1994) and more recently cyanobacterial strain PCC 6803 (Liu et al. 2011) and high concentrations of fatty acid have been shown to accumulate as a result of the absence of feedback inhibition of acyl-CoA's, but only when combined with alterations that overcome other metabolic bottlenecks in fatty acid production (Lu et al. 2008).

1.7 Fatty acid degradation

β-oxidation is the major degradative metabolic pathway for short- and long-chain fatty acids in living organisms. In addition to β-oxidation, there are also the minor metabolic pathways α-oxidation and ω-oxidation. α-oxidation is concerned with the degradation of phytol, a component of chlorophyll. The methyl group on carbon 3 of phytol prevents β-oxidation occuring, so the α-carbon is oxidized instead by phytanoyl-CoA dioxygenase, converting it to pristanic acid that can then enter the β-oxidation pathway (Jansen and Wanders, 2006). Phytanoyl-CoA dioxygenase is only present in some types of proteobacteria and eukaryotes, and is missing in most bacteria. ω-oxidation is concerned with the biosynthesis of dicarboxylic acids, such as pimelic acid, a precursor to biotin that is outlined in 1.6 and traumatin, a compound involved in wound response in plants (Zimmerman and Coudron, 1979). It is not specifically known whether cyanobacteria carry out ω-oxidation, but it is prevalent in many organisms by cytochrome P450 enzymes acting upon fatty acids and converting them to dicarboxylic fatty acids via a hydroxyl intermediate (Van Bogaert et al. 2010), these enzymes are highly conserved (Vanhanen et al. 2000)

The β-oxidation pathway has been known for a long time (Dakin, 1909), and the mapping of this pathway employed one of the earliest examples of metabolite tracing, decades before heavy isotopes and radioisotopes were available. Franz Knoop, a German chemist, fed dogs fatty acids that were derivatised with a phenyl ring, subsequent analysis of the dogs' urine samples revealed that the metabolic end products were different depending on whether the chain length of the fatty acid was odd or even, detecting phenylacetic acid and benzoic acid respectively; from this he was able to deduce that initial oxidation occurred on the β carbon and that with each round of oxidation the acyl chain of the fatty acid was being decreased in length by 2 carbons (Knoop, 1904). This later led to the discovery of fatty acid biosynthesis in the

1940s, with the feeding of the 2 carbon compound acetate to mice revealing its incorporation into fatty acids by stable isotope labeling (Rittenberg and Bloch, 1945).

An overview of β-oxidation is given in Fig 1.5. The first and probably most important step in this pathway with regards to regulation of β-oxidation, involves the activation of the fatty acid to an acyl-CoA, catalysed by acyl-CoA synthetase; in *E. coli* this enzyme is encoded by *fadD* (Overath, 1969) – termed *oldD* in this publication, both old and new gene names are given in the following description. This reaction proceeds via a two-step mechanism involving the hydrolysis of ATP to pyrophosphate to drive the endergonic reaction, forming a fatty acyl adenylate intermediate, the carboxyl group of which undergoes nucleophilic attack from the thiol sulphur of CoA-SH, forming the end product acyl-CoA plus AMP and pyrophosphate. This reaction would be readily reversible if it were not for the conversion of pyrophosphate to 2 P_i by pyrophosphatase, resulting in the direction of the reaction lying far to the right.

The remainder of the pathway is essentially the reverse of fatty acid biosynthesis, except that the acyl moiety is bound to coenzyme A instead of acyl carrier protein (ACP). The first step of β-oxidation is an oxidation/dehydrogenation step, where the acyl-CoA thioester is oxidised by acyl-CoA dehydrogenase, the product of *fadE* (*yafH*), forming a trans-Δ^2-enoyl-CoA. This is then hydrated by enoyl-CoA hydratase (*fadB* gene (*oldB*)) to an L-β-hydroxyacyl-CoA, which is then oxidised again by β-hydroxyacyl-CoA dehydrogenase (*fadJ* gene (*yfcX*)) to form β-ketoacyl-CoA. This is then cleaved between the α and the β carbons by acyl-CoA acetyltransferase to give acetyl-CoA, and another CoA group is ligated to the β carbon of the remainder of the acyl chain. This new acyl-CoA thioester can then undergo the same series of reactions again in a cyclical manner. Thus if an acyl-CoA thioester that contains 16 carbon atoms undergoes 7 rounds of β-oxidation, it will yield 8 molecules of acetyl-CoA.

It is generally assumed that β-oxidation is a universally-occurring metabolic pathway, and this pathway would make a good target for downregulation in order to improve

yields of hydrocarbons in cyanobacteria. However, evidence for the presence of this pathway in the cyanobacteria is currently lacking, both in the literature and by genome annotation. The *fadD* ortholog, which is annotated as an acyl-CoA synthetase according to the public sequence database Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000), has been shown to be an acyl-ACP synthetase, this was demostrated experimentally by Kaczmarzyk and Fulda (2010). Two unusual strains of cyanobacteria, *Cyanothece* PCC 7425 and *Acaryochloris marina* appear to have all β-oxidation enzymes present according to KEGG, although none of these enzymes in these organisms have been experimentally verified. These strains are somewhat unusual for cyanobacteria as, PCC 7425 has been observed to have unusual structural features, such as a high content of light refractile inclusions, concentric cortical thylakoids, a compact central nucleoid (Porta et al. 1999), as well as an unusual pattern of cell division compared to other cyanobacteria, and *A. marina* is unusual as it contains chlorophyll *d* (Marquardt et al. 1997), which uses far-red light at 714 to 718 nm (Miyashita et al. 1996).

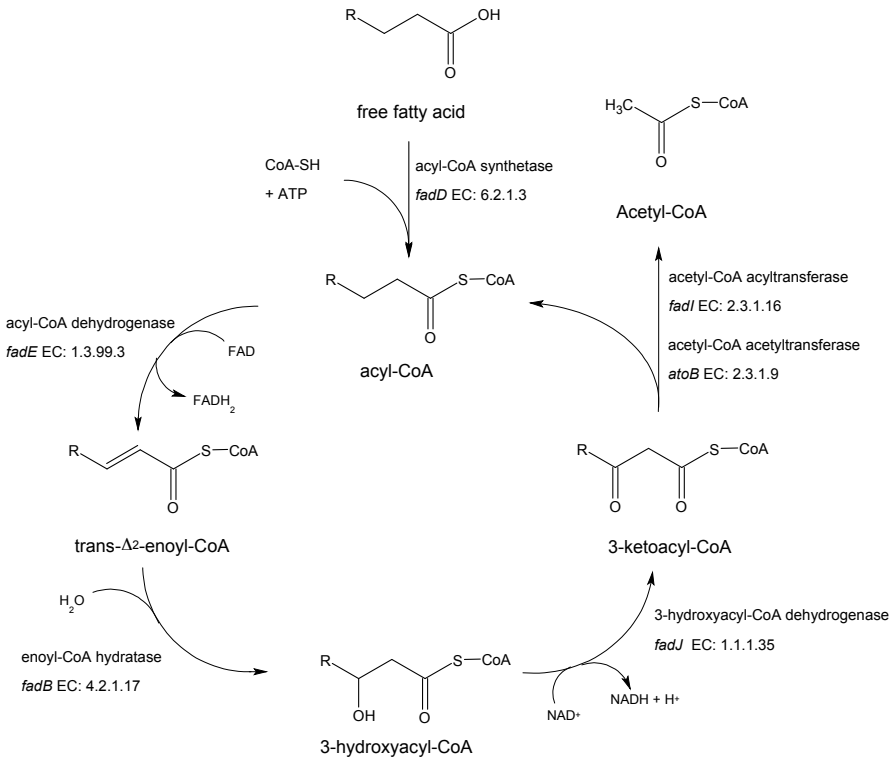

Figure 1.5. Overview of β-oxidation. Free fatty acid is activated to an acyl-CoA thioester by acyl-CoA synthetase, whereby it can then enter a series of 4 cyclic oxidation reactions to form acetyl-CoA. Acyl-CoA is converted to an enoyl-CoA by dehydrogenation of carbon 2 (the β-carbon) and carbon 3 of the acyl chain by acyl-CoA dehydrogenase, introducing a carbon-carbon double bond. C=C bond is hydrated to form an –OH group on carbon 3 by enoyl-CoA hydratase to give 3-hydroxyacyl-CoA. This –OH group is dehydrogenated by 3-hydroxyacyl-CoA dehydrogenase to form 3-ketoacyl-CoA. This keto group can then be attacked by acyl-CoA acyltransferase to liberate one acetyl-CoA molecule and thus shorten the acyl chain length by 2 carbons. This cycle occurs until only acetyl-CoA remains. For the final thiolytic cleavage of acetoacetyl-CoA to 2 acetyl-CoA's, an acetyltransferase instead of acyltransferase is used.

1.8 Fatty acid homeostasis

In addition to modifying the flux of carbon into fatty acids via gene overexpression or knock-out/knock-down, knowing and manipulating the mechanisms that naturally regulate the cells' internal fatty acid pool could prove to be a more effective way of improving the flux of carbon into these compounds.

The manner in which fatty acid biosynthesis and degradation are regulated greatly depends on the type of organism. In eukaryotes the regulation is generally much more complex than in prokaryotes as they have cell compartmentation, which serves as an extra regulatory element. In animals for example, the mitochondria are the site of β-oxidation and acyl-CoAs need to be transported into the mitochondria. The acyl-CoA is transported across the outer mitochondrial membrane through an acyl-CoA carrier protein and in the periplasmic space is converted into an acyl-carnitine by carnitine acyltransferase I, so that it can cross the inner mitochondrial membrane that only accepts acyl-carnitines through a specific carrier protein (Bremer, 1983). Once inside the mitochondrial matrix it is converted back into an acyl-CoA for β-oxidation. Carnitine acyltransferase I is inhibited by malonyl-CoA, an intermediate of fatty acid biosynthesis (see section 1.6), so under conditions that favour fatty acid synthesis, β-oxidation is prevented from occurring as acyl-CoAs cannot reach the site where this occurs. Likewise, transportation of acetyl-CoA out of the mitochondria relies on a complex membrane shuttling system that involves acetyl-CoA being converted to citrate by reacting with oxaloacetate by citrate synthase before being converted back to oxaloacetate and acetyl-CoA by citrate lyase (Chappell, 1968). Of course cyanobacteria lack such compartmentation so this type of regulation is not applicable to these organisms. Hormones also play a large part in fatty acid homeostasis in animals, which are probably not applicable to cyanobacteria either as long-range chemical communication is not needed, although this could still be relevant to

filamentous strains or cyanobacteria that grow as part of a biofilm, where quorum sensing plays an important role in biofilm stability (Shapiro, 1998).

One key enzyme in which fatty acid biosynthesis is regulated in bacteria, plants and other eukaryotes appears to be the acetyl-CoA carboxylase complex (see 1.6). In eukaryotes the polymerisation of this dimer to its active form is inhibited by the presence of free acyl-CoAs and promoted by the presence of citrate (Munday, 2002); this enzyme has a long half-life within the cell and so is not regulated at the transcriptional level. In plants, both eukaryotic and prokaryotic isoforms of this enzyme are present (Alban et al. 1994) and it is thought that a similar mechanism could regulate the eukaryotic isoform (Ohlrogge and Jaworski, 1997), although it is not currently known what specific (ant)agonists are responsible for this regulation. The prokaryotic isoform of acetyl-CoA carboxylase found in the plastids of plants is regulated solely via direct feedback inhibition by acyl-ACPs (Andre et al. 2012).

E. coli represents the most studied bacterial organism in the field of fatty acid homeostasis. Other well-studied bacteria are the model gram-positive *Bacillus subtilis* and the pathogenic *Streptococcus pneumoniae* (Fujita et al. 2007). Regulation of fatty acid biosynthesis in bacteria has been found to operate predominantly at the transcriptional level, modulated by regulatory proteins. In *E. coli*, 2 transcriptional regulators have been identified; FadR and FabR.

FadR acts as both a repressor of β-oxidation (*fad*) genes and a promoter of the fatty acid biosynthesis genes *fabA* and *fabB*, involved in the synthesis of unsaturated fatty acids in *E. coli*. When repressing, this protein binds to -30 and +10 of the start site of the *fad* genes, blocking the binding of RNA polymerase to the start codon of the gene (Henry and Cronan, 1992), at the same time it promotes expression of biosynthetic enzymes *fabA* and *fabB* by binding to a 17 bp region found 40 bp upstream of their respective start sites that aids recruitment of RNA polymerase. FadR is itself directly inhibited by binding of long-chain acyl-CoAs, which causes a derepression of the *fad*

genes. So under conditions of excess acyl-CoA, the substrates of β-oxidation, binding of this compound to FadR occurs, resulting in a change in the regulators' conformation so that it can no longer bind to the -30 and +10 regions of the *fad* genes, releasing the regulator and allowing transcription of β-oxidation genes to occur. As well as regulating β-oxidation and fatty acid biosynthesis, FadR also represses transcription of *uspA* as well as *yhcX* and *yhcY* genes, and promotes the expression of *iclR*. UspA (universal stress protein) is upregulated under a wide array of stress conditions such as starvation, heat shock, osmotic shock and the presence of toxins (Nystrom and Neidhardt, 1994). *yhcX* and *yhcY* are involved in the anaerobic degradation of fatty acids (Campbell et al. 2003). IclR represses the transcription of *aceBAK* operon (Maloy and Nunn, 1981), which are genes encoding enzymes of the glyoxylate cycle that are involved in the synthesis of carbohydrates from acetyl-CoA. This type of regulatory protein is found in some members of γ-proteobacteria only (Raman et al. 1997).

E. coli FabR is another transcriptional regulator found in *E. coli* that is concerned with the regulation of unsaturated fatty acid biosynthesis, repressing *fabA* and *fabB* (Zhang et al. 2002). FabA is responsible for introducing a *cis*- C=C of β-hydroxydecanoyl-ACP as well as the *trans*-configuration normally observed in the reaction carried out by FabZ for the synthesis of saturated fatty acids. *fabB* encodes KAS I that is also required for production of unsaturated fatty acids, probably as it is the only KAS able to accept decenoyl-ACP (Magnusson et al. 1993). A homolog of this regulator has also been found in *Pseudomonas aeruginosa* (Zhang et al. 2005). The signal that modulates the activity of FabR is currently unknown.

Not only does *E. coli* regulate at the transcriptional level, but also by direct feedback inhibition in the same manner as eukaryotic organisms. Acetyl-CoA carboxylase, FabH and FabI are inhibited by acyl-ACPs (Heath and Rock, 1996). This is likely to give a more rapid response than that of transcriptional repression to ensure that excessive amounts of fatty acids are not synthesized. At the same time transcriptional repression

is probably more essential in a unicellular organism as opposed to a multicellular one like an animal or plant as protein synthesis also uses up limiting resources that are valuable to the organism.

A FabR homolog is present in *B. subtilis*, which interacts with 15 genes in 5 operons involved in β-oxidation (Matsuoka et al. 2007) - note that these homologs in *E. coli* are not arranged into operons. The enzyme is inhibited by acyl-CoAs much in the same way that *E. coli* FadR is inhibited. In addition to FadR, another regulator, FapR, is responsible for regulating the transcription of all the fatty acid biosynthesis genes. This regulator has 6 targets, 3 of which are in an operon, with binding sites found in the promoter regions of these operons (Schujman et al. 2003). Binding of this protein is inhibited by malonyl-CoA; therefore under conditions favouring fatty acid biosynthesis where high concentrations of malonyl-CoA are present, derepression occurs. Both FapR and *B. subilis*-type FadR are highly conserved amongst a wide range of gram-positive bacteria and even some proteobacteria and archaea. There is also a possibility that acetyl-CoA carboxylase is also directly inhibited by acyl-ACPs in the same manner as *E. coli* and other eukaryotes (Fujita, 2007), although evidence for this is currently lacking.

In *S. pneumoniae* the regulation of β-oxidation is less well characterized. However the regulation of fatty acid biosynthesis is regulated by a transcriptional repressor, FabT. FabT represses the transcription of all of the fatty acid biosynthetic genes (Lu and Rock, 2006) except *fabM*, which are arranged in a single operon. *fabM* is involved in unsaturated fatty acid biosynthesis, but acts after the trans-enoyl-ACP has been formed, unlike in *E. coli*. It was found that this repressor binds downstream of the *fabM* start site, but upstream of all the other fatty acid biosynthetic genes. Similar gene clusters containing all of the fatty acid biosynthetic genes have been found in a range of other bacteria including *Enterococcus, Clostridium* and *Lactococcus.* The signal that

modulates the activity of this regulatory protein is still not known, or whether direct inhibition of fatty acid biosynthetic enzymes occurs.

In addition to these specific regulatory mechanisms, fatty acid biosynthesis and β-oxidation are also influenced by the more global regulatory systems that are found in bacteria, such as the cyclic-AMP/receptor system and the stringent response. In conditions of carbon starvation such as low glucose concentration in heterotrophic bacteria, levels of cAMP rise and bind to the cAMP receptor protein, converting it to its active form, which then can promote the transcription of a range of genes involved in obtaining energy from other carbon sources (Pastan and Perlman, 1970) including β-oxidation genes (Pauli et al. 1974). The stringent response is activated when the cell is starved of amino acids resulting in free tRNAs. When a free tRNA enters the A-site of the ribosome, protein synthesis is stalled, activating the ribosome bound RelA protein, which synthesizes the nucleotide guanosine-3',5'-bispyrophosphate (ppGpp) (Chatterji and Ojha, 2001). This acts as a signaling molecule that can control many areas of gene expression at the transcriptional level, stopping growth and inducing a stress response (Magnusson et al. 2005). The stringent response is well characterized in a number of species of bacteria and in the chloroplasts of plants (Masuda et al. 2008) so cyanobacteria are very likely to have such a mechanism. The stringent response is also activated by inhibition of fatty acid biosynthesis; ACP interacts with the TGS domain of the SpoT protein, SpoT can both degrade or synthesize ppGpp depending upon its conformation. Under conditions of fatty acid starvation, free holo-ACP can change the conformation of SpoT, favouring its ppGpp synthesis activity, and so creating a stringent response (Battesti and Bouveret, 2006).

One final area of fatty acid biosynthesis regulation that has been touched on already is unsaturated fatty acid biosynthesis. As mentioned above, this is carried out by specific fatty acid biosynthesis enzymes encoded by *fabA* and *fabB* in *E. coli* and by *fabM* in *S. pneumoniae*, with analogous systems present in many other bacteria. In cyanobacteria

and eukaryotes, unsaturated fatty acids are synthesized by a class of desaturase (Des) enzymes that act upon the acyl chain (Los and Murata, 1998). In animals and fungi, desaturases act upon acyl-CoAs, whereas in plants and cyanobacteria the desaturases act upon fatty acids that are incorporated into membrane lipids. Additionally plant desaturases also act upon ACPs. *B. subtilis* has both the bacterial and Des systems of saturated fatty acid biosynthesis (Aguilar et al. 2001). The expression and activity of Des genes and proteins is greatly influenced by temperature, as a greater degree of unsaturation in a lipid results in it having a lower melting point and so these enzymes tend to be activated at lower temperatures in order to keep the cell membranes in a fluid state. Overexpression of these enzymes could have a potential industrial benefit for synthesis of unsaturated hydrocarbon production in cyanobacteria, as a low melting temperature is useful for motor fuels, for example hexadecane melts at approximately 17 °C, whereas hexadecene melts at 4 °C.

1.9 Aims and the scope of the project

One of the key areas that was apparent when reviewing the literature on cyanobacterial fatty acid metabolism was the absence of any reports outlining the lack of a β-oxidation pathway in cyanobacteria. An observation that suggests that this pathway may not be present was that no homologs of β-oxidation enzymes appeared to be present on the KEGG database for all but 2 strains (see 1.7).

Therefore the hypothesis that cyanobacteria do not have an active β-oxidation pathway was tested (3.1). Additional confirmation was sought by cloning an element of the β-oxidation pathway into a cyanobacterial strain and detecting its' resulting activity (3.2), as well as analysing one of the strains that has all of the β-oxidation genes present according to homology (3.3). Finally the regulation of fatty acid biosynthetic pathways was determined, as a lack of β-oxidation means that biosynthesis must be tightly regulated if its end product cannot be degraded (3.4).

2. Materials and Methods

2.1 Chemicals used

Chemicals for general use were purchased from Fisher (Loughborough), Sigma-Aldrich (Gillingham), VWR (Lutterworth), BD Biosciences (Oxford), LabM (Bury), AGTC Bioproducts (Hessle), QMX (Thaxted) and American Radiolabeled Chemicals (Stevenage) unless otherwise stated.

2.2 Cyanobacteria strain information

Table 2.1. Cyanobacteria strains used

Species	Strain number	Source
Synechocystis sp.	PCC 6803	Pasteur culture collection
Synechocystis sp. "glucose tolerant"	PCC 6803 GT-S	Obtained from Dr David Lea-Smith, University of Cambridge
Nostoc sp.	PCC 7120	Pasteur culture collection
Anabaena variabilis	PCC 7937	Pasteur culture collection
Acaryochloris sp.	HICR111A	Gift from Professor Wolfgang Hess, University of Freiburg, Germany

2.2.1 Growth conditions

All microbiological work was carried out under sterile conditions. Strains PCC 6803, PCC 7120 and PCC 7937 were grown in 100 ml sterile BG-11 medium according to Rippka et al. 1979. HICR111A was grown in sterile artificial seawater (ASW)/ BG-11 1:1 mix according Mohr et al. 2010. All strains were grown in sterile 250 ml conical flasks, incubated in an illuminated incubator shaker at 28 °C, 3.2 % CO_2, shaking at 125 rpm and 80 μmol m^{-2} s^{-1} photon flux density.

Cyanobacterial cultures were maintained at an exponential growth rate by subculturing 5 ml of culture at mid to late exponential phase ($OD_{730\ nm}$ = 8) into 100 ml fresh liquid BG-11 medium.

For growth on solid media, PCC 6803, PCC 7120 and PCC 7937 were grown on BG-11 1.5% w/v agar plates. PCC 6803 transformants were grown on BG-11 agar plates supplemented with 50 μg ml^{-1} kanamycin.

BG-11 medium

Stock 1 – 100x BG-11

$NaNO_3$	149.58 g l^{-1}
$MgSO_4$. $7H_2O$	7.49 g l^{-1}
$CaCl_2$. $2H_2O$	3.6 g l^{-1}
10X Trace metal mix	100 ml l^{-1} (v/v)

Stock 2 – HEPES buffer

HEPES	238 g l^{-1}
Adjust to pH 7.8 with 10 M NaOH	

Stock 3 – Ferric ammonium citrate (FAC) + citric acid monohydrate

FAC	1.2 g l^{-1}
Citric acid . H_2O	1.2 g l^{-1}

Stock 4 – Sodium carbonate

Na_2CO_3	20 g l^{-1}

Stock 5 – Potassium phosphate dibasic

K_2HPO_4	30 g l^{-1}

Stock 6 – Ethylenediaminetetraacetic acid dipotassium magnesium salt (IDRANAL) (Fluka)

IDRANAL	0.2 g l^{-1}

Volumes of stock solutions required for 1 L of BG-11 medium

Stock 1	10 ml
Stock 2	5 ml
Stock 3	5 ml
Stock 4	1 ml
Stock 5	1 ml
Stock 6	5 ml
Water	to 1 l

10 X Trace metal mix (added to Stock 1)

H_3BO_3	2.86 g l^{-1}
$MnCl_2$. $4H_2O$	1.8 g l^{-1}
$ZnSO_4$. $7H_2O$	0.22 g l^{-1}
Na_2MoO_4 . $2H_2O$	0.39 g l^{-1}
$CuSO_4$.$5H_2O$	0.079 g l^{-1}
$Co(NO_3)_2$. $6H_2O$	0.0494 g l^{-1}

Artificial Sea Water (ASW)

NaCl	28.1 g l^{-1}
$CaCl_2$.$2H_2O$	1.48 g l^{-1}
KCl	0.68 g l^{-1}
$MgSO_4$.$7H_2O$	7.9 g l^{-1}
$MgCl_2$.$6H_2O$	5.48 g l^{-1}

ASW was mixed 1:1 with BG-11 for growth of HICR111A.

BG-11 agarose

300 ml BG-11 was autoclaved and mixed with 300 ml autoclaved 3 % w/v agarose to give a final concentration of 1.5% w/v agarose, cooled to approximately 55 °C, appropriate antibiotic was added if required and plates poured. This is enough to pour 20 plates.

2.2.2 Cryopreservation

Cyanobacteria

Cryopreservation was necessary for long-term storage of cyanobacterial cultures (Starr and Zeikus, 1993). 1 ml of culture was pelleted by centrifugation at 5000 *g* for 5 min and resuspended in 1 ml sterile 5 % methanol in BG-11 medium and transferred to a -80 °C freezer where it is allowed to cool slowly. Thawing of stocks was done rapidly in a water bath at 37 °C, cells were pelleted by centifugation at 3000 *g* for 5 min and resuspended in 1 ml fresh BG-11 medium then kept in the dark for 2 days to recover. After this period, stocks can be subcultured into fresh BG-11 medium and grown as described in 2.2.1.

E. coli

Transformants and vector-expressing *E. coli* were stored long term as glycerol stocks at -80 °C. 800 μl *E. coli* culture was added to 200 μl 80 % glycerol in a cryovial and flash frozen in liquid nitrogen. Stocks were then transferred to a -80 °C freezer for storage. Recovery was carried out by scraping off some culture from the cryovial with a sterile pipette tip and spreading on an LB-agar plate, with appropriate antibiotic selection if necessary.

2.3 Growth conditions of *E. coli*

The following *E. coli* strains were used:

- DH5α (genotype: F- ϕ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 thi-1 gyrA96 relA1 λ-) (Invitrogen)
- DB3.1 (genotype: F- gyrA462 endA1 glnV44 Δ(sr1-recA) mcrB mrr hsdS20(r_B^-, m_B^-) ara14 galK2 lacY1 proA2 rpsL20(Sm^r) xyl5 Δleu mtl1) (Invitrogen)
- TOP10 (genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG λ-) (Invitrogen)
- DH10B (genotype: F- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *end*A1 *ara*D139 Δ (*ara*, *leu*)7697 *gal*U *gal*K λ- *rps*L *nup*G /pMON14272 / pMON7124)

For the purpose of obtaining genomic DNA for amplification of *fadD* by PCR and for use as positive control in the acyl-CoA assay and enzyme assays, DH5α cells were spread on LB-agar plates and grown overnight at 37 °C. Single colonies were then picked from the plates and inoculated into 15 ml LB liquid broth and grown overnight, shaking at 125 rpm at 37 °C. Strain DB3.1 was utilised for propagating plasmids that contain the DNA gyrase toxin gene *ccdB*. The strain was transformed as described in 2.15.2 and single colonies of transformants grown as described above. DH10B cells were used to propagate conjugal and helper plasmids pRL443 and pRL623, strain DH10B cells were used as this strain lacks the *mcr* and *mrr* restriction systems that

digest DNA containing methylcytosine and methyladenine (Raleigh et al. 1988; Blumenthal, 1989). For the purpose of propagating other plasmids, TOP10 cells were transformed as described in 2.15.1, single colonies were grown as described above with the addition of antibiotic in order to select for the plasmid, with single colonies being grown as described above, also with the addition of appropriate antibiotic.

LB liquid broth (Bertani, 1951):

Tryptone	10 g L^{-1}
Yeast extract	5 g L^{-1}
NaCl	10 g L^{-1}

LB agar:

As above, supplemented with 15 g L^{-1} agar

When selecting for various plasmids, a range of antibiotics were used at the following final concentrations:

Antibiotic	Concentration
Ampicillin	100 μg ml^{-1}
Chloramphenicol	25 μg ml^{-1}
Kanamycin	50 μg ml^{-1}

2.4 Use of oxygen electrode

Oxygen concentration of cell cultures was measured by a Hansatech DW2/2 oxygen electrode, which is controlled by Oxylab v1.15 software. 2 ml of culture was added and illuminated by an array of red LED's of variable intensity.

Photon flux density (PFD) was controlled by the software, starting from an intensity of 0 μmol m^{-2} s^{-1} and increasing up to an eventual intensity of 660 μmol m^{-2} s^{-1} for 5 minutes at each PFD. Rate of oxygen evolution was measured in corresponding 5-minute time segments. Rates were measured as n=3 and normalised according to OD.

2.4.1 Measurement of respiration rates of *E. coli* and cyanobacteria on a range of carbon sources

PCC 6803 and PCC 7120 from the illuminated incubator and light starved samples that were kept in the dark for 12 hours prior to analysis, but in otherwise normal conditions, were tested, along with culture of *E. coli* as positive control. All strains were incubated with a low concentration of appropriate carbon source at 0.2 mM concentration for an hour prior to testing in order to induce that specific metabolic pathway (if present).

Carbon sources in their solvent were made up to a stock concentration of 10 mM (Table 2.2). 200 μl of this was then spiked into 1.8 ml of cyanobacterial sample, giving a working concentration of 1 mM. All measurements were conducted in dark conditions. Initial rate of respiration was recorded before spiking in the carbon source for a period of 5 min (Fig. 2.1, A), after which the carbon source was spiked in at a working concentration of 1 mM. After 2 min (Fig. 2.1, B), the rate of respiration was measured again for 5 min (Fig. 2.1, C). From this second measurement of respiration rate, the initial respiration rate was subtracted, and the blank that the carbon source is dissolved in is subtracted to give the true induced rate of respiration of the culture on that specific carbon source. The rate data obtained was then normalized by dividing the rate values against culture OD.

Table 2.2. Carbon sources spiked into cultures. Acyl chain length (if applicable), and which solvent stock solutions were dissolved in.

Carbon Source	Acyl chain length	Solvent
Glucose	n/a	Water
Acetate	2 carbon	Water
Valeric acid	5 carbon	Water
Decanoic acid	10 carbon	Ethanol
Palmitic acid	16 carbon	Ethanol
Oleic acid	18 carbon unsaturated	Ethanol
Stearic acid	18 carbon	Ethanol

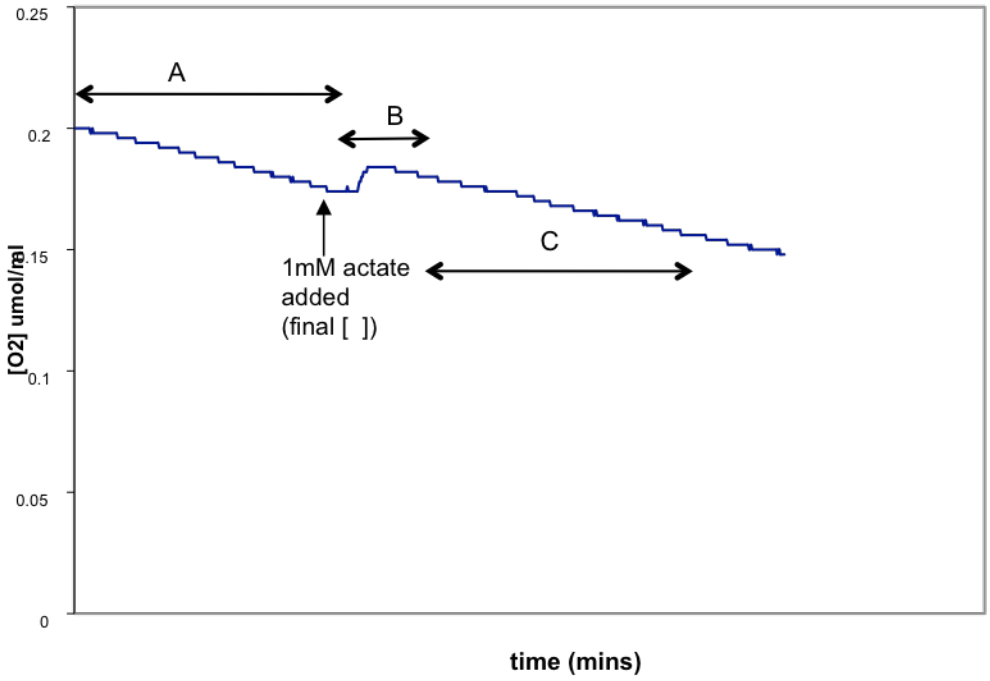

Figure 2.1. Feeding strategy in oxygen electrode. **A.** Initial rate of oxygen consumption (respiration) is taken from the slope. **B**. After spiking in of carbon source (in this case acetate), no measurement of rate is taken for 2 minutes, as addition of the carbon source or removal of the electrode chamber lid can cause a momentary increase in dissolved oxygen in the sample. **C.** Rate of oxygen consumption with the carbon source present is measured from the slope.

2.5 Detection of Acyl-CoAs in cell extracts

2.5.1 Preparation of Acyl-CoA standards

5 mg of the following acyl-coenzyme A standards were purchased from Sigma-Aldrich: acetyl-CoA, palmitoyl-CoA (16:0-CoA), palmitoleoyl-coA (16:1-CoA) and stearoyl-CoA (18:0-CoA). These were made up to 5 mM stocks and working concentrations of 75 μM (150 pmol total acyl-CoA in a 2 µl injection volume) were used for method development and analysis.

2.5.2 Extraction, SPE and enrichment of acyl-coAs

2 ml liquid cultures of PCC 6803, PCC 7120, PCC 7937 and HICR111A at an OD_{730} of 4, as well as positive control *E. coli* at an OD_{600} of 4 were harvested by centrifugation, freeze dried overnight and ground into a powder using a mortar and pestle. Acyl-CoAs were extracted in acetonitrile and isopropanol 3+1, v+v in a tissue homogeniser (MP Bio FastPrep 24), settings 6.0 m/s, 40 sec. Acyl-CoAs were enriched by solid-phase extraction (SPE) using 2-(2-pyridyl)ethyl-functionalized silica gel columns (Supelco) according to Minkler et al. 2008.

2.5.3 Method development of liquid chromatography-triple quadrupole-mass spectrometry (LCMS-QQQ)

Standards and extracts were separated using an Agilent 1200 Series HPLC running a C18 2.1 x 30 mm 3.5-μm reverse-phase column. Autosampler temperature was kept at 4 °C, with an injection volume of 2 μl of sample. Mobile phase pumped through the column at a rate of 0.3 ml min^{-1}. Acyl-CoAs were eluted isocratically, using a method adapted from Veld et al. 2009; 55% LCMS grade acetonitrile (ACN) + 45% 10 mM ammonium acetate, pH 6.7 (NH_4Ac) was pumped through at 100% for 3 min. In order to eliminate the possibility of carryover contamination, the sample injection needle was washed in 55% ACN + 45% LCMS grade water in the instrument flush port for 3 s, and the column was flushed with 95% ACN + 5% NH_4Ac for 12 min. Total run time was 15 min for each sample.

MS2, product ion and multiple reaction monitoring (MRM) scans were performed using an Agilent 6410 Series triple quadrupole mass spectrometer with electrospray ionization (ESI) at 4000 V, both positive and negative polarities were tested. Fragmentor voltage was set to 135 V and skimmer set to 70 V, drying gas flow temperature was 350 °C and flow rate 10 l min^{-1}. Scan time for MRMs was 500 ms.

2.6 Bioinformatic analysis of β-oxidation enzymes in cyanobacteria

KEGG (Kanehisa and Goto, 2000) (http://www.genome.jp/kegg) was used to check which enzymes are involved in the β-oxidation pathway of 40 fully sequenced cyanobacteria. Each of the following enzymes involved in beta-oxidation were checked: acyl-CoA synthetase (EC:6.2.1.3); acyl-CoA dehydrogenase (EC:1.3.99.-; 1.3.99.3; 1.3.99.13); enoyl-CoA hydratase (EC:4.2.1.17; 4.2.1.74); 3-hydroxyacyl-CoA dehydrogenase (EC:1.1.1.35; 1.1.1.211); acetyl-CoA acyltransferase (EC:2.3.1.16).

For each of the listed enzymes, BLAST searches were performed of known cyanobacterial sequences against other uncharacterized cyanobacterial genomes. Significant hits (score >80, E-value < 1e-15) were analysed by BLAST searching against the NCBI microbe BLAST database and using Pfam software (Finn et al. 2010) to search for conserved domains. Alignments were made using CLC Sequence Viewer 6 (CLC Bio).

2.7 Feeding of fatty acids to cyanobacteria and *E. coli*

20 ml of water, 10 % DMSO in water (working conc.), 10 % ethanol in water (working conc.), 5 % ethanol in water (working conc.) or 1 % ethanol in water (working conc.) was incubated with 180 ml PCC 6803, PCC 7120 and *E. coli* strain DH5α. These solvents were completely miscible with the culture medium. Other solvents not soluble in culture medium were also tested; isobutanol, 1-pentanol, 1-octanol, hexane, nonane, decane, tetradecane and tetradecene. These solvents formed a separate phase that sat on top of the culture medium.

After this period, viability was assessed by inoculating 5 ml of fed cyanobacteria culture into 100 ml fresh liquid BG-11 or 0.5 ml fed *E. coli* culture into 10 ml LB broth and OD measured at 730 nm for cyanobacteria or 600 nm for *E. coli*.

2.8 Gas chromatography mass spectrometry (GCMS)

2.8.1 Sample preparation

100 ml cyanobacteria culture was centrifuged at 3900 *g* and washed 3 times in LCMS grade water to remove residual exogenous fatty acids that were fed to cultures and freeze dried. 15 mg of dry cell material was homogenised in a mortar and pestle using acid washed sand to disrupt cells and lipids were extracted in dichloromethane (DCM) using Dionex ASE150 accelerated solvent extraction system (Thermo Scientific). This extract is dried down under N_2 gas and resuspended in 1.5 ml DCM with 5 μg ml^{-1} d_8-napthalene as an internal standard for quantification.

2.8.2 Derivatisation

Two derivatisation methods were tested, 1-ethyl-3,3-dimethylaminopropylcarbodiimide (EDC) that generates methyl esters and the silating agent *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA).

1. Derivatisation using EDC

For standards, 100 μl of 10 mg ml^{-1} fatty acid in DCM (1mg total) was dried down under N_2 gas and re-dissolved in 400 μl methanol. 10 μl of 100 mg ml^{-1} (10 mg total) EDC was added and incubated for 2 h at 25 °C. The reaction was stopped with 200 μl 0.1 M tris-HCl, pH 7.5. fatty acid methyl esters (FAMEs) were extracted by washing twice with 1 ml hexane. Extracts in hexane were then dried under N_2 gas and redissolved in 100 μl DCM.

2. Derivatisation using BSTFA

10 μl BSTFA was added to 100μl of 10 mg ml^{-1} fatty acid (1mg total) and incubated at 80 °C for 30 min.

Both reactions were tested by TLC (2.9.6) to determine if derivatisation was successful before analyzing standards and samples by GCMS.

2.8.3 GCMS parameters

GCMS data were acquired using a ThermoQuest Finnigan Trace GC 2000 series using a Rtx-5MS 15 m x 0.25 mm x 0.25 μm GC column (Restek) connected to a ThermoQuest Finnigan Trace MS with electron impact ionization, with a scanning range of 30-700 m/z. Samples were injected by an AS2000 autosampler with a volume of 2 μl splitless injection. The instrument was controlled using Xcalibur v1.1 acquisition software.

Initial temperature of the GC column was at 35 °C for 2 min, increasing by 10 °C min^{-1} until it reached 320 °C, this temperature was held for 5 minutes. Carrier gas was helium with a flow rate of 1.0 ml min^{-1}. Total run time for each sample was approximately 35 minutes. Peaks were identified by comparison with known standards, retention time and comparisons of their mass spectra with the NIST database. Quantification of alkanes was based on heptadecane standards.

2.9 Radiolabelling

2.9.1 Feeding

Acetate

5 μCi of 1,2-^{14}C-acetate (American Radiolabeled Chemicals Inc., specific activity 100 mCi mmol^{-1}) was fed to 10 ml culture with an OD of 4 (730 nm for cyanobacteria and 600 nm for *E.coli*) for 4 h either under a light intensity of 60 μmol m^{-2} s^{-1} or in the dark, both with and without unlabeled carrier at 1 mM concentration in order to determine whether this assists uptake of acetate. *E. coli* were incubated at 37 °C and cyanobacteria were incubated at 28 °C. No CO_2 was supplemented to the cyanobacteria (atmospheric CO_2 concentration).

Fatty acids – Pulse chase

10 ml cultures of cyanobacteria and *E. coli* at OD = 4 were fed 1-^{14}C-decanoic acid (American Radiolabeled Chemicals Inc.) at a concentration of 10 μCi (specific activity 50 mCi mmol^{-1}) in 5 ml of culture for 1 h in the dark to prevent cyanobacteria re-using any CO_2 that may have become labeled with ^{14}C from decanoic acid by photosynthesis. *E. coli* were incubated at 37 °C and cyanobacteria were incubated at 28 °C. No CO_2 was supplemented to the cyanobacteria (atmospheric CO_2 concentration).

$^{14}CO_2$ was trapped using a filter paper wick soaked with 1 M potassium hydroxide in a chamber of the sealed culture vessel (Medinsky, 1986). After 1 h, a 1 ml sample was harvested by centrifugation and cultures were resuspended in label-free culture medium. Additional 1 ml samples were harvested at 4 h, 8 h and 20 h. At all timepoints, the KOH wick was removed and counted with a scintillation counter as described in 2.9.7.

2.9.2 Fractionation

The culture was fractioned into the following components: culture medium; aqueous phase; lipid phase and pellet.

Cells were harvested by centrifugation at 4000 *g* and resulting supernatant (culture medium) was dried down under a stream of N_2 gas overnight, resuspended in 200 µl methanol/water 2:1 v/v, 20 µl of which was counted in a liquid scintillation counter with 3 ml scintillation cocktail added (Sigma-Aldrich).

The resulting pellets were resuspended in 3.75 ml MeOH/dichloromethane 2:1 v/v and incubated for 30 min, shaking at 780 rpm. To achieve phase separation, 1.25 ml water and 1.25 ml DCM were added after incubation. The lower DCM phase was carefully removed with a glass Pasteur pipette and dried down under a stream of N_2 gas. The dried residue was resuspended in 200 µl DCM, 20 µl of which was counted in a scintillation counter and 20 µl aliquots were spotted onto each TLC plate.

The remaining upper aqueous phase was centrifuged and supernatant dried down overnight under a stream of N_2 gas, resuspended in 200 µl MeOH/H_2O 2:1 v/v, 20 µl of which was counted in a scintillation counter and aliquots of 20 µl spotted onto TLC plates. The entire remaining pellet from aqueous phase was counted in a scintillation counter (2.9.7).

2.9.3 Ion exchange chromatography

Ion exchange chromatography was performed based on the method described by Redgewell, 1979. Anion exchange columns (Strata SAX, 100 mg/1 ml) and cation exchange columns (Strata SCX, 100 mg/1 ml) were both solvated with 4 ml methanol, followed by 4 ml water. The pH of aqueous phase was adjusted from pH 5.5 to pH 3 by dropwise addition of 2 M formic acid to protonate amino acids and allow them to bind to the functional $–SO_3^-$ group on the cation exchange column. All of the aqueous phase was loaded to the cation exchange column and washed three times with 1 ml water. The cation exchange column was washed three times with 1 ml 6 M NH_4OH to elute cationic compounds (e.g. amino acids).

The initial flowthrough from the cation exchange column was then loaded onto the anion exchange column and washed three times with water to elute neutral compounds (e.g. sugars). Column was then washed with 2 M formic acid to elute anionic compounds (e.g. sugar phosphates, organic acids) from the functional $–N^+$ group of the anion exchange column; 200 μl of each resulting fraction is transferred to scintillation medium for counting (2.9.7).

2.9.4 Silver (Ag^+) ion chromatography

Supelco Discovery Ag Ion SPE 5 ml columns were solvated with 5 ml acetonitrile followed by 5 ml acetone followed by 5 ml DCM. DCM phase was dried down and resuspended in 1 ml DCM, which was loaded onto the column and the flowthough collected. The column was washed with 5 ml DCM to elute alkanes, esters, aldehydes into the second fraction. 5 ml 10% acetone in DCM was used to elute alkenes, acids (including fatty acids), diols and epoxides into the third fraction. 5 ml acetone was used to elute the remaining compounds (e.g. complex lipids) into the final final fraction. All fractions except the final fraction were dried down and resusupended in 250 μl DCM, 50 μl of which is spotted onto a silica TLC plate, and 200 μl of which was transferred to liquid scintillation medium for counting (2.9.7).

2.9.5 Column chromatography of complex lipids

This protocol has been adapted from Pernet et al. 2006. The final fraction eluted from the Ag^+ ion column was loaded onto a Strata SI-1 Silica column (100 mg/1 ml) that had been solvated with 10 ml Methanol, followed by 10 ml acetone, followed by 10 ml DCM. Initial flowthough was collected. Complex neutral lipids such as triacylglycerides (TAGs) were eluted with 10 ml DCM. Glycolipids were eluted by adding 15 ml 10% acetone in methanol. The most polar phospholipids were eluted with 10 ml methanol. All elutions were dried down and neutral lipids resuspended in 50 μl DCM; glycolipid and phospholipid fractions were resuspended in 50 μl 2:1 v/v DCM/Methanol. These fractions were spotted onto a silica TLC plate.

2.9.6 Thin layer chromatography

Compounds were separated on Alugram SIL G silica TLC plates. 20 μl samples of cell fractions from 2.9.2 were applied to the plate 1.5 cm from the base and with 1 cm distance from the sides and between each sample and allowed to dry. The plate was ran in a saturated environment in a TLC tank using the appropriate solvent system for a length of 15 cm, dried under the fume hood and lipids were visualized by the appropriate method.

Solvent systems

Three solvent systems were employed depending on compound class:

1. Ethanol / 16.1 M ammonium solution 7:3 v/v for aqueous phase and culture medium.

2. Petroleum ether / DCM / acetic acid 90:10:1 v/v/v for separating alkanes and alkenes from fatty acids and from complex lipids in the lipid phase.

3. Acetone / toluene / water 90:30:8 v/v/v for separating complex lipids.

Staining methods

Four stains were used to visualize where standards had migrated to on the TLC plate. Stains were applied to TLC plates using a compressed air powered chromatography spray gun in a fume hood.

1. Primuline – Lipids were detected by staining with 0.05 % primuline w/v in acetone/water 8:2 v/v, allowing to air dry and visualizing under short wave UV light.

2. Ninhydrin – Amino acids were detected by staining with 0.25 % w/v ninhydrin in acetone, allowing stain to briefly air dry and heating at 60 °C for 10 min.

3. Bromophenol blue – TLC plates were heated to 60 °C for 30 min to evaporate all ammonia from the mobile phase. Plates were then stained with 0.1 % w/v bromophenol blue and air dried to visualize organic acids and sugars.

4. Iodine – TLC plate was placed in a warm TLC tank with iodine vapour for 10 min to visualize compounds that contain carbon-carbon double bonds. This method was used to detect alkenes.

Standards

The standards listed in table 2.3 were used at a concentration of 10 $\mu g\ \mu l^{-1}$. 2 μl was spotted onto an appropriate TLC plate (20 μg of standard in total spotted).

Autoradiography

In order to visualize where activity from 1,2-^{14}C-acetate and 1-^{14}C-decanoic acid was incorporated into compounds of interest, TLC plates were wrapped in cling film to prevent contamination and incubated with X-ray film in a cassette at -80 °C for 48 h, after which the film was developed in an Optimax film processor at 30 °C, with developing, fixing, washing and drying steps of 20 s each.

Table 2.3 List of standards from a range of compound classes. Listed are the solvent systems they were tested on and which staining method was employed.

Compound	Class	Solvent systems tested	Staining method
Hexadecene	Hydrocarbon	All	4
Palmitic acid	Fatty acid	All	1
Digalactosyl diacylglycerol	Glycolipid	2 and 3	1
Glyceryl tripalmitate	TAG	2 and 3	1
Sucrose	Sugar – disaccharide	1	3
Fructose	Sugar – monosaccharaide	1	3
Amino acid standard mix*	Amino acids	1	2
Citric acid	Organic acid	1	3
Succinic acid	Organic acid	1	3
Fumaric acid	Organic acid	1	3
Malic acid	Organic acid	1	3
Acetic acid	Organic acid	1	3
Methyl palmitate	FAME	2	1

2.9.7 Scintillation counting

20 μl of each fraction from 2.9.2 was added to 2 ml scintillation cocktail and disintegrations per minute (DPM) were counted on a Beckman Coulter LS 6500 multipurpose scintillation counter. Samples were counted for 5 minutes each.

2.9.8 Analysis of spots using liquid chromatography-quadrupole time of flight-mass spectrometry (LC-QToF-MS)

In order to identify any compounds of interest showing up as spots on autoradiograms where radioactivity from labeled decanoic had become incorporated, cultures were fed unlabeled decanoic acid in parallel and the same procedures carried out as described in 2.9.2.

TLC and extraction

TLC was performed as described in 2.9.6 and development of autoradiograms, corresponding spots on unlabeled TLC plates were excised by scraping and extracted in 100% methanol for analysis by LC-QToF-MS.

LC-QToF-MS parameters

Samples were separated on an Agilent 1200 Series HPLC running a 100 x 2.1 mm C18 reverse phase column (1.8 µm particle size) mobile phase was 50% ACN, increasing to 95% after 25 min, with 3 min re-eqilibration time. Samples were ionised by ESI, both in negative and positive polarities, with a capillary voltage of 3500 V, fragmentor set to 115 V, skimmer 70 V and drying gas at a temperature of 325 °C with a flow rate of 9 l min^{-1}.

MS/MS data obtained using an Agilent 6520 Accurate Mass QToF mass spectrometer controlled using MassHunter Acquisition software. The instrument was set to scan in the mass range of 100 to 2000 m/z in MS mode and 40 to 3000 m/z in MS/MS mode. Collision energy was determined according to the following equation:

collision energy = gradient x m/z / 100 + offset

Where gradient was set to 3.6 V and offset was set to -4.8 V. In order to avoid MS/MS being performed on dominant ions in every MS/MS scan, active exclusion was used after recording 2 spectra, and released after 0.1 min. Precursor ion minimum threshold

was set at 1000 counts. The instrument scanned in mass range of 300 to 2500 m/z at 5 scans s^{-1} for MS mode and in the mass range of 50 to 3000 m/z at 4 scans s^{-1} for MS/MS mode. Analysis of samples and identification of compounds was performed using Agilent MassHunter 4 Qualitative Analysis software.

2.10 Protein extraction from cells

Protein extraction methods were different depending upon application of extracted protein. Below are listed extraction methods for protein extraction from cyanobacteria for proteomics, protein extraction from *E. coli* or cyanobacteria for enzyme assays and protein extraction from cress seedlings.

2.10.1 Protein extraction for proteomics

2 ml of cyanobacterial culture was harvested by centrifugation at 7500 g for 5 min at 4 °C, pellet was resuspended in 2 ml protein extraction buffer (20 mM Tris-HCl, pH 7.8; 1 mM EDTA; 2 mM dithiotheritol (DTT)). Cells were disrupted by 5 cycles in a tissue homogeniser (MP Bio) at 6.5 m/s, 30 s, before being spun down at 400 g for 5 min. Protein concentration was determined by the Bradford assay (2.10.4).

2.10.2 Protein extraction for enzyme activity assays

10 ml liquid cultures of cyanobacterial cultures at $OD_{730\ nm}$ = 4 and *E. coli* $OD_{600\ nm}$ =4 were harvested by centrifugation at 3000 *g* for 10 min. Pellets were resuspended in 4 ml protein extraction buffer (20 mM Tris-HCl, pH 7.8; 1 mM ethylenediaminetetraacetic acid (EDTA); 200 μl 1x protease inhibitor cocktail (Roche) and 2 mM dithiothreitol (DTT)). *E. coli* were disrupted with 6x 10 sec pulses at 50% amplitude using a sonicating needle, cyanobacteria were disrupted by bead-beating with 50 to 70 mesh sand, 4x 40 sec pulses at 6.5 m s^{-1}, due to their stronger cell walls (Jurgens et al. 1983). Cell debris was removed by centrifugation and the resulting crude protein extracted in the supernatant was quantified by the Bradford assay (2.10.4). Cells and

extracts were kept on ice or chilled to 4 °C throughout the procedure to avoid loss of enzyme activity through denaturation.

2.10.3 Protein extraction from cress

As a positive control for acyl-CoA oxidase assays, 2 day old cress seedlings (*Lepidium sativum*) were ground in a pestle and mortar in liquid nitrogen, 4 ml extraction buffer (2.10.2) was added and the resulting slurry was disrupted with 6x 10 sec pulses at 50% amplitude using a sonicating needle. Cell debris was removed by centrifugation and the resulting crude protein extracted in the supernatant was quantified by the Bradford assay (2.10.4).

2.10.4 Protein quantification

The Bradford assay (Bradford, 1976) was used to determine the protein concentration in the extracts prepared in 2.10.1, 2.10.2 and 2.10.3. 10 µl of protein sample or standard was added to 200 µl Bradford reagent diluted with 790 µl water and mixed. After 10 min incubation at RT, the absorbance at 595 nm was measured vs. water. A standard curve was prepared by measuring the absorbance of the following BSA standards: 0 mg ml^{-1}, 0.25 mg ml^{-1}, 0.5 mg ml^{-1}, 0.75 mg ml^{-1}, 1 mg ml^{-1} and 1.5 mg ml^{-1}.

2.11 Tryptic digests

4 µg of extracted protein was used for the digest. 4 µl 10 mM DTT was added to sample and incubated at 50 °C for 20 min to derivitise –SH groups on cysteine residues to prevent formation of tertiary structure. Sample was incubated in the dark for 20 minutes with 4 µl 50 mM iodoacetamide (IAA) and 2µl 500 mM ammonium bicarbonate. 0.1 µg trypsin was added and incubated at 37 °C for 16 h. The reaction was stopped by adding 1 µl 1 % formic acid. Sample was cleaned up by centrifugation

at 16100 g for 30 min, and supernatant transferred to an autosampler vial for proteomic analysis by LC-QToF-MS.

2.12 Proteomics using nano-ESI-LC-QToF-MS

Protein samples were ran on an Agilent 1200 Series HPLC and 6520 Accurate Mass Q-ToF LC/MS. Proteins were separated on a high capacity enrichment chip with 150 mm C18 analytical column. Ionisation was by nano-ESI-LCMS running in positive ion mode using a capillary voltage of 1850 V, drying gas temperature of 300 °C at a flow rate of 4 l min^{-1}, fragmentor set to 150 V and skimmer at 70 V. Collision energy was determined by the same method as described in 2.9.8, with a gradient of 3.6 V and offset of -4.8 V. Injection volume 0.3 μl. Solvent A was 2 % LCMS grade methanol and 0.1 % formic acid; Solvent B was 5 % methanol and 5 % formic acid. Active exclusion and precursor threshold values were the same as described in 2.9.8. The instrument scanned in the mass range of 300 to 2500 m/z in MS mode and 50 to 3000 m/z in MS/MS mode. Total run time for each sample was 97 minutes. Instrument was controlled by MassHunter Data Acquisition software. Analysis was carried out using Spectrum Mill proteomics data mining software (Agilent).

2.13 Enzyme activity assays

All spectrophotometric measurements were performed in a Shimadzu UV2101-PC UV-Vis scanning spectrophotometer in quartz cuvettes with a 1 cm light path.

2.13.1 Acyl-CoA dehydrogenase assay

Acyl-CoA dehydrogenase activity was measured at 37 °C, following the rate of reduction of iodonitrotetrazolium (INT) as measured by the increase in absorbance at 492 nm. The 1 ml reaction mixture contained 0.25 mM INT, 0.1 mM phenazine methosulfate (PMS), 0.15% triton x-100, 10 μM FAD and 100 μl crude protein extract (2.10.2) in buffer consisting of 20 mM Tris-HCl, pH 7.8 and 1 mM EDTA. All

components of reaction mixture were gassed with N_2 to remove atmospheric O_2, and the reaction cuvette was sealed with an air-tight bung. The mixture was allowed to stabilise in the spectrophotometer before the substrate, 0.1 mM 16:0-CoA, was spiked in using a 20 μl syringe. Activity was measured from the initial rate, using an extinction coefficient of 19.4 mM^{-1} cm^{-1} (Dommes and Kunau, 1976).

2.13.2 Acyl-CoA oxidase assay

Acyl-CoA oxidase activity was measured at 37 °C, activity was determined by measuring the production of H_2O_2 by coupling with peroxidase and following the oxidation of pyrogallol to purpurogallin at 430 nm. The 1 ml reaction mixture contained 5 U peroxidase, 4 mM pyrogallol, 100 μl crude protein extract (2.10.2), 0.2 mM NAD, 12 mM DTT, 0.15 mg ml^{-1} and triton X100 0.01% in buffer consisting of 30 mM HEPES, pH 7.4. The mixture was allowed to stabilise in the spectrophotometer before the substrate, 0.1 mM 16:0-CoA, was spiked in using a 20 μl syringe. Activity was measured from the initial rate, using an extinction coefficient (ε) calculated to be 3.99 $mM^{-1}cm^{-1}$.

2.13.3. Acyl-CoA synthetase assay

Acyl-CoA synthetase activity was based on a method described by Rock and Cronan, 1981. The assay was carried out in 1.5 ml microcentrifuge tubes in a volume of 40 µl. The assay mixture contained 100 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 5 mM ATP, 2.5 mM DTT, 0.5 mM CoA, 2% Triton X-100, 30 µM 1-^{14}C-lauric acid and a defined amount of crude protein extract. The reaction was initiated by addition of the protein sample and incubated for 2 h at 37°C. The assay was stopped by transferring the whole reaction mixture to a paper filter disc. The filters were dried and washed twice with DCM/ methanol/ acetic acid (3 : 6 : 1) free fatty acids The filter discs were dried again and counted in 3 ml of scintillation cocktail.

2.14 Cloning of *E.coli fadD* into *Synechocystis* PCC 6803

Cloning of *E.coli fadD* was carried out using the BioBrick method (Shetty et al. 2008).

2.14.1 Genomic DNA extraction

Genomic DNA from cyanobacteria and *E.coli* was extracted using GenElute gDNA kit (Sigma-Aldrich). For cyanobacteria, the gram positive extraction protocol was followed and for *E.coli* the gram negative extraction protocol was followed.

For extraction of genomic DNA from gram positive bacteria, 2 ml of cells were harvested by centrifugation at 13,000 *g* for 2 min, cell wall was digested by resuspending the pellet in 200 μl lysozyme solution and incubating at 37 °C for 30 min. Cells were lysed by the addition of 20 μl of proteinase K with 200 μl of its lysis buffer at 55 °C for 10 min. 200 μl was added to the lysed cells and added to a supplied binding column, which was centrifuged at 6500 *g* for 1 min to bind the DNA to the column. The column was washed twice with 500 μl of wash solution to remove contaminants by centrifuging at 6500 *g* for 3 minutes. DNA was eluted in 200 μl 10 mM tris-HCl, pH 9.0, 100 mM EDTA by centrifuging at 6500 *g* for 1 min. For extraction of genomic DNA from gram negative bacteria, the same protocol was followed, but without the cell wall digestion step.

2.14.2 PCR

The polymerase chain reaction (PCR) was used for the amplification of specific DNA sequences. This method is based on cyclic repetition of thermal DNA denaturation, hybridization with specific primers and extension by a thermostable DNA polymerase, which results in exponential increase of the desired sequence (Mullis and Faloona, 1987). When amplifying sequences for use in cloning, a proofreading DNA polymerase (Phusion polymerase) was used as the number of errors introduced in these sequences needed to be minimal. For diagnostic PCRs, *Taq* polymerase was used. The reactions were carried out in a G-Storm thermocycler (G-Storm).

2.14.3 PCR of *fadD* from *E. coli*

fadD was amplified from *E. coli* using the primers listed below. Biobrick site containing appropriate restriction sites were added to the 5' end of the primer.

fadD forward primer:

AAAGAATTCGCGGCCGCTTCTAGATGAAGAAGGTTTGGCTTAACCGTTATC

fadD reverse primer:

AAACTGCAGCGGCCGCTACTAGTATTATTAGGCTTTATTGTCCACTTTGCCG

Key:

EcoRI

SpeI

PstI

XbaI

Hybridising region

BioBrick site

Reaction Mix (50 µl total volume):

Phusion DNA polymerase (New England Biolabs)	0.5 µl
5x Phusion HF buffer	10 µl
dNTPs, 10mM	1 µl
forward primer, 10 µM	2.5 µl
reverse primer , 10 µM	2.5 µl
template DNA	2 µl
nuclease free water	31.5 µl

PCR Program:

Step	Temperature	Duration	
Initial denaturation	98 °C	30 s	
Denaturation	98 °C	10 s	30 cycles
Annealing	64 °C	30 s	
Extension	72 °C	1 min	
Final extension	72 °C	10 min	

10 μl aliquots of each reaction were checked they were the expected size on an agarose gel. The remaining 40 μl was stored at -20 °C until required for subsequent steps in cloning.

2.14.4 PCR of promoter sequences from *Synechocystis* and primer purification

Primers used in a study by Huang et al. 2010 were used to amplify the promoter sequence of ribulose bisphosphate carboxylase subunit *rbcL*. These primers were designed to bind to sequences 267 bp for the forward primer and 18 bp for the reverse primer upstream from the start codon of *rbcL*. Below are listed the primers for amplification of these sequences:

rbcL forward primer:

TGGAATTCGCGGCCGCATCTAGAGTCACCATTTGGACAAAACATCAGCAATTC

rbcL reverse primer:

AGCCTGCAGCGGCCGCTACTAGTATTTCTCCTCTTTAAACATTGAATA

Key:

EcoRI

SpeI

PstI

XbaI

Hybridising region

BioBrick site

Additionally, these primers required further purification in order to improve the efficiency of the PCR. (http://molbio.mgh.harvard.edu/szostakweb/protocols/denaturepage/index.html) Primers were precipitated in 100 % ethanol at -80 °C for 20 min, centrifuged for 10 min at 4°C, pellet washed with 70 % ethanol and lyophilized to dryness. Primer was resuspended in urea loading buffer consisting of 8 M urea, 20 mM EDTA, 5mM Tris-HCl, pH 7.5 and 0.5 % w/v bromophenol blue and heated to 90 °C for 5 min. Primers were then purified on a 20 % polyacrylamide denaturing gel containing 7 M urea and ran at 200 V until the bromophenol marker had migrated 75 % of total gel length. Bands containing primer were visualised under UV light, excised from the gel and pureed using a narrow bore syringe. 3 ml of TE buffer for every 0.5 ml of pureed gel was added and the sample frozen by incubating at -80 °C after which the sample was rapidly thawed at 90 °C for 5 min. Purified primers were allowed to elute overnight on a rotary shaker. Gel was removed from primers by centrifugation and filtration through a 0.2 μm syringe filter. Primer was concentrated by liquid-liquid phase separation using *n*-butanol, aqueous phase was removed and 0.3 M sodium acetate, pH 5.2 and 2 volumes of 100 % ethanol was used to precipitate the primer at -20 °C for 30 min. Primer was pelleted by centrifugation, 16,000 *g* for 10 min and then redissolved in 250 μl TE buffer.

Reaction Mix (50 μl reaction):

Phusion DNA polymerase	0.5 μl
5x Phusion HF buffer	10 μl
dNTPs, 10mM	1 μl
forward primer, 10 μM	2.5 μl
reverse primer , 10 μM	2.5 μl
template DNA	2 μl
DMSO 1.25% v/v	0.625 μl
nuclease free water	31.5 μl

PCR Program:

Step	Temperature	Duration	
Initial denaturation	98 °C	30 s	
Denaturation	98 °C	10 s	35 cycles
Annealing	61 °C	30 s	
Extension	72 °C	25 s	
Final extension	72 °C	10 min	

10 μl aliquots of each reaction were checked they were the expected size on an agarose gel. The remaining 40 μl was stored at -20 °C until required for subsequent steps in cloning.

2.14.5 Diagnostic and quality control PCR's

For the purpose of checking the successful insertion of DNA fragments into plasmids and for sequencing, verification primers that bind to sites typically 90 – 125 bp upstream (VF2) and 145 – 165 bp downstream (VR) from the BioBrick sites were used (BioBrick part numbers BBa_G001 and BBa_G00101 respectively). For the purpose of checking that homologous recombination 1 site had been successfully inserted into expression vector pPMQAK1, a verification primer that binds 96 bp upstream of the site was designed (VF3GT), as the VF2 site is downstream from this region. Additionally,

the other primers for *rbcL* and *fadD* (2.15.2.1 and 2.15.2.2) were also used for verification. The verification primer sequences are listed below:

Forward verification primer (VF2): CCACCTGACGTCTAAGAAAC

Reverse verification primer (VR): GTATTACCGCCTTTGAGTGA

Reaction Mix (20 μl):

Taq DNA polymerase (New England Biolabs)	0.1 μl
10x Taq buffer	2 μl
dNTPs, 10mM	0.4 μl
forward verification primer, 10 μM	0.4 μl
reverse verification primer, 10 μM	0.4 μl
template DNA	0.4 μl
nuclease free water	16.3 μl

PCR Program:

Step	Temperature	Duration	
Initial denaturation	95 °C	30 s	
Denaturation	95 °C	10 s	} 30 cycles
Annealing	54 °C	30 s	
Extension	68 °C	variable	
Final extension	68 °C	5 min	

In some cases, it was quicker to check clones by performing a colony PCR, in which case the following reaction mix and PCR program were used:

Reaction Mix (20 μl):

Taq DNA polymerase (New England Biolabs)	0.1 μl
10x Taq buffer	2 μl
dNTPs, 10mM	0.4 μl
forward verification primer, 10 μM	0.4 μl
reverse verification primer, 10 μM	0.4 μl
nuclease free water	16.7 μl

A single colony from plated clones was picked using a pipette tip and mixed into the above reaction mix before being spread onto a new LB-agar plate with appropriate antibiotic for selection present.

PCR Program:

Step	Temperature	Duration	
Initial denaturation	95 °C	5 min 30 s	
Denaturation	95 °C	10 s	30 cycles
Annealing	54 °C	30 s	
Extension	68 °C	variable	
Final extension	68 °C	5 min	

2.14.6 Plasmids

Plasmids pSB1A3 and pSB1C3 were obtained from the Registry of Standard Biological Parts (partsregistry.org). Plasmid pPMQAK1 (Huang et al. 2010) was kindly provided by Dr Thorsten Heidorn (Uppsala University). pSB1A3 and pSB1C3 contained the RFP gene in the BioBrick site, allowing for negative selection as colonies where ligations were unsuccessful would fluoresce red. pPMQAK1 contained DNA gyrase toxin-encoding suicide gene *ccdB*; this plasmid was propagated in *E. coli* strain DB3.1. Plasmids pRL443 and pRL623 that were required for triparental mating were kindly provided by Professor Peter Wolk (Michigan State University).

Table 2.4. List of plasmids used for cloning and transformations.

Plasmid	Selection	Other elements
pSB1A3	Ampicillin	Terminators bracketing MCS Verification primer binding sites
pSB1C3	Chloramphenicol	Terminators bracketing MCS Verification primer binding sites
pPMQAK1	Ampicillin and kanamycin	Terminators bracketing MCS Verification primer binding sites *Rep* genes for maintenance within cyanobacterial host *Mob* genes for conjugational transfer
pRL623	Chloramphenicol	Contains AvaI and AvaII methylases to prevent restriction of DNA in cyanobacterial hosts
pRL443	Ampicillin	RP4 based conjugal plasmid

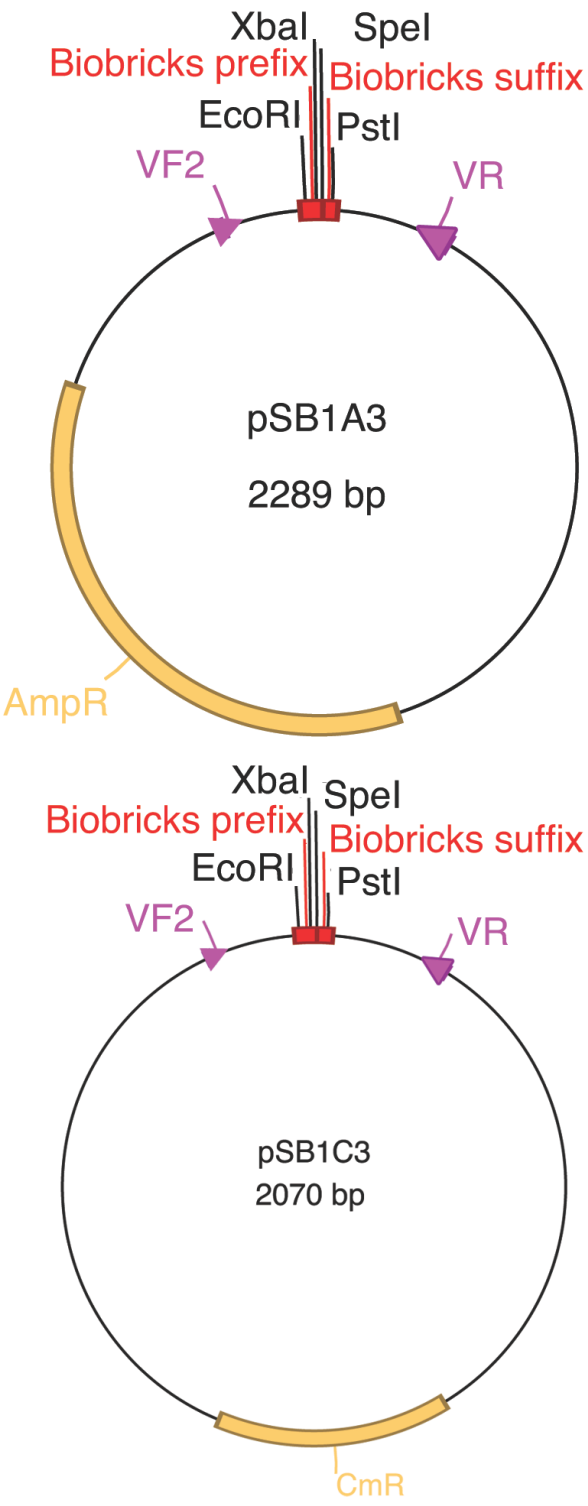

Figure 2.2. Plasmid maps of pSB1A3 and pSB1C3. Resistance cassettes, primer binding sites and restriction sites within the BioBrick prefix and suffix are shown.

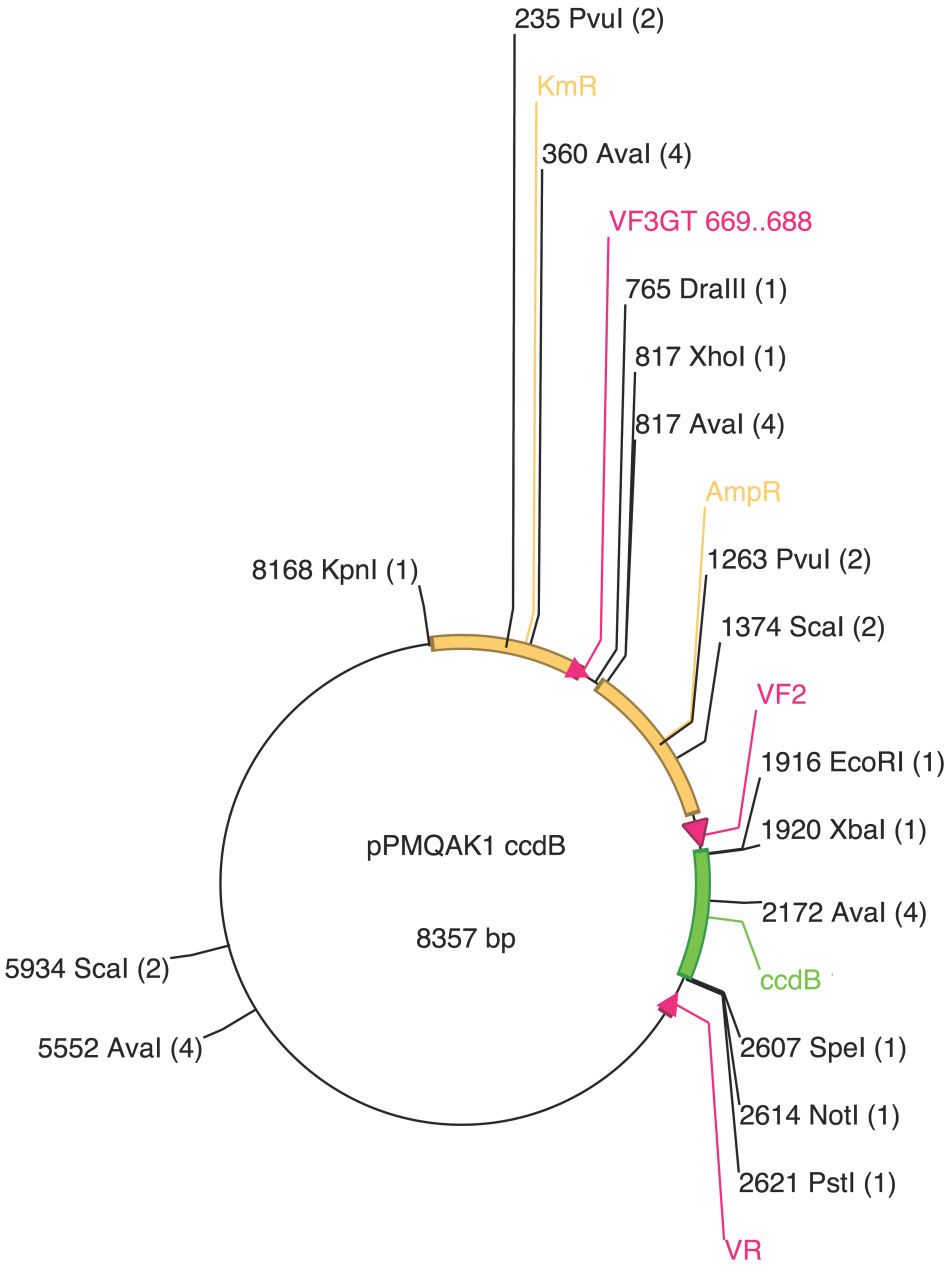

Figure 2.3. Plasmid map of pPMQAK1. Resistance cassettes, primer binding sites, *ccdB* gene and important restriction sites are shown. Suicide gene *ccdB* is flanked by BioBrick prefix and suffix MCS containing restriction sites EcoRI, XbaI, SpeI, NotI and PstI. *Mob* and *Rep* genes not shown.

2.14.7 Plasmid DNA preparation

Desired plasmid DNA was extracted and isolated from 15 ml overnight *E. coli* cultures using QiaPrep Spin Miniprep kits (Qiagen). Preparation was performed according to manufacturers protocol.

2.14.8 DNA cleanup

Following certain reactions such as restriction digests it was necessary to purify the DNA from other components of the reaction mixture as they interfere with downstream processes. This was achieved using NBS Spin Column Reaction Cleanup kit (NBS).

2.14.9 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments made during restriction digests and PCR. Depending upon size of DNA fragment separated, different strength gels were made; For separating very small fragments such as the *rbcL* promoter (253 bp) a 1.5 % w/v agarose gel was used, for other purposes either a 1 % or 0.8 % gel was used. Agarose was melted in TAE buffer using a microwave oven, cooled to approximately 50 °C and 0.001 % v/v of 10 mg ml^{-1} ethidium bromide solution was added to the gel, and the gel poured and allowed to set. DNA samples were mixed with 1/6 volume TriTrack DNA loading dye (Fermentas) before being loaded into the gel wells. 5 μl HyperLadder I (Bioline) was loaded into at least 1 well and used as a size marker. Gels were run at 100 V for 45 min in TAE running buffer. DNA bands were visualized and photographed under UV light using a transilluminator.

TAE buffer, 1 L:

Tris base	4.84 g
Acetic acid	1.142 g
0.5 M EDTA	2 ml

2.14.10 DNA excision from agarose gels

DNA was excised and isolated from agarose gels using NBS Spin Column Gel Extraction kit (NBS Biologicals). Procedure was carried out according to manufacturers instructions.

2.14.11 Determination of concentration of nucleic acids

The concentration of DNA and RNA was determined by measuring the absorbance at 260 nm using a NanoDrop ND1000 spectrophotometer (Thermo Scientific). 50 ng μl^{-1} of DNA has an A_{260} of 1 and 40 ng μl^{-1} of DNA has an A_{260} of 1, the concentration is calculated by the software.

2.14.12 Cloning strategy

1. *fadD* and *rbcL* promoter sequences were amplified from *E.coli* and PCC 6803 by PCR.

2. Quality control 1: PCR products were checked they were of correct size on agarose gel.

3. RFP was cut out of BioBrick site in pSB1A3 & pSB1C3 using restriction enzymes EcoRI and PstI.

4. Quality control 2: It was determined whether RFP had been excised from plasmids by running digest on an agarose gel. Empty plasmid was excised from gel.

5. *fadD* was ligated into pSB1A3 and *rbcL* promoter ligated into pSB1C3. Ligation was transformed into *E. coli* strain TOP10. TOP10 was grown on LB-agar plates containing appropriate antibiotic to select for transformants.

6. Quality control 3: Successful ligations were screened 1. Visually as colonies where ligations were unsuccessful fluoresced red due to RFP still being present in the BioBrick site. 2. By a diagnostic PCR to determine if inserts are of correct size. 3. By diagnostic restriction digest using NotI to determine if bands of the expected size are observed. 4. By sequencing to ensure that no mutations had been introduced.

7. Expression vector was assembled. *rbcL* promoter was cut from pSB1C3 using restriction enzymes EcoRI and SpeI, *fadD* was cut from pSB1A3 using restriction enzymes XbaI and PstI. *ccdB* was excised from BioBrick site of pPMQAK1 using EcoRI and PstI. All 3 digests were ligated and transformed into TOP10. TOP10 were grown on LB-agar plates containing appropriate antibiotic to select for transformants.

8. Quality control 4: Successful ligations were screened by diagnostic PCRs to determine if inserts were of correct size. 3. By diagnostic restriction digest using NotI to

determine if bands of the expected size were observed. 4. By sequencing to ensure that no mutations had been introduced.

9. pPMQAK1 containing *rbcL* promoter and *fadD* was transformed into PCC 6803.

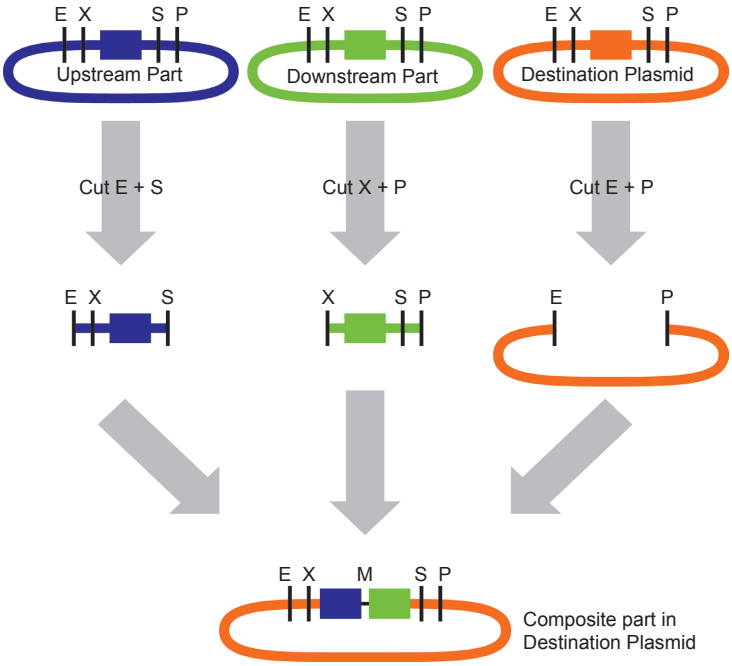

Figure 2.4. Schematic overview of BioBrick assembly. Where Upstream Part is pSB1C3 containing *rbcL* promoter sequence (dark blue); Downstream Part is pSB1A3 containing *fadD* (green); Destination Plasmid is pPMQAK1 containing *ccdB* (orange). E = EcoRI, X = XbaI, M = mixed site, S = SpeI, P = PstI. Figure adapted from Ginkgo Bioworks assembly manual (ginkgobioworks.com).

2.14.13 Restriction digests

Restriction enzymes recognize a specific sequence of nucleotides and produce a cut on both strands of dsDNA. In all cases 2 μg DNA was digested with 2 U of appropriate restriction enzyme (Promega or NEB). The reaction was performed according to manufacturers protocol.

2.14.14 Dephosphorylation

Shrimp alkaline phosphatase (Promega) catalyses the removal of phosphate groups from the 5' end of the DNA molecule, reducing the possibility of self-ligation of plasmids following a restriction digest. DNA was cleaned up according to 2.15.5 and mixed with 1 U of shrimp alkaline phosphatase per μg of DNA in phosphatase reaction buffer and incubated for 15 min at 37 °C and heat inactivated for 20 min at 65 °C.

2.14.15 Ligations

T4 DNA ligase (Fermentas) was used to ligate desired DNA fragments into plasmids by generating covalent phosphodiester bonds between 3' hydroxy and 5' phosphate ends of double stranded DNA fragments. For generation of pSB1A3 and pSB1C3 vectors containing *fadD* and *rbcL* promoter, the following formula was used to calculate the total amount of DNA fragment to be inserted into 50 ng total of vector:

$$\text{ng insert} = \frac{\text{50 ng vector x size of insert (kb) x 3}}{\text{size of vector (kb)}}$$

Ligation reaction was performed according to manufacturers instructions for 60 min at 25 °C. The mixture was then transformed into TOP10 *E. coli* according to 2.15.14.1. For generation of expression vector pPMQAK1 with *rbcL* promoter and *fadD*, the 20 μl ligation reaction mix consisted of the following:

T4 DNA ligase	1 μl
10 x ligase buffer	2 μl
rbcL promoter cut with EcoRI and SpeI	2 μl
fadD cut with XbaI and PstI	2 μl
pPMQAK1 cut with EcoRI and PstI	2 μl
water	11 μl

The reaction was incubated for 10 min at 25 °C and inactivated for 20 min at 80 °C. The mixture was then transformed into TOP10 *E. coli* according to 2.15.14.1.

2.14.16 Sequencing of vectors

Plasmids were sequenced based on the method originally described by Sanger et al. (1977) by GATC Biotech (GATC Biotech Ltd.). 30 to 100 ng μl DNA was used. The verification primers VF2 and VR (2.15.2.4) were used for checking vector sequence. Additionally, so all of the *fadD* gene insert could be sequenced in pPMQAK1, a third primer with the sequence GTTGTATACCCCGCGTGA was designed to bind 316 bp into fadD for the short version and 383bp into the long version of the gene.

2.15 Transformation methods

2.15.1 *E. coli* heat-shock transformation

50 μl aliquots of desired *E. coli* strain (TOP10 or DB3.1) was removed from -80 °C storage and thawed on ice. 2 μl of DNA from the ligation reaction to be transformed was added to the cells and incubated on ice for 30 min. The cells were heat shocked at 42 °C for 30 s before being put back on ice for a further 2 min. After this period 250 μl pre warmed super optimal broth (SOB) (Invitrogen) was added to the cells and incubated, shaking at 225 rpm at 37 °C for 1 hr. After this period the transformations were spread on LB-agar plates containing the appropriate antibiotic for selection and grown overnight at 37 °C.

2.15.2 Transformation of cyanobacteria by triparental mating

Cyanobacteria were transformed by the “spot mating” method of triparental conjugation as described by Elhai and Wolk (1988).

pPMQAK1 (cargo plasmid) was transformed into *E.coli* strain DH10B carrying the helper plasmid pRL623 by the heat-shock method as described in 2.17.1 and grown on LB agar overnight with appropriate antibiotic selection. Additional confirmation of both plasmids being in DH10B was given by 1. Screening for cargo plasmid using PCR to amplify the region between verification sites. 2. Screening for plasmid pRL623 by miniprepping plasmid DNA and checking for a band on the gel at 9 Kb.

750 μl overnight cultures of DH10B containing pPMQAK1 and pRL623, and of *E. coli* strain HB101 containing pRL443 were washed and resuspended in 60 μl LB containing no antibiotic. 1.5 ml of mid- to late- log phase cyanobacterial cultures were resuspended in 100 ul BG-11. A 5 μl spot of cyanobacteria was applied to a sterile nitrocellulose filter on a BG-11 agar plate. To each of these spots, 2 μl of the *E. coli* mixture was applied and mixed thoroughly with a pipette tip. The plate was then transferred to a static light incubator for 24 h at optimal conditions for cyanobacterial growth. After 24 h the nitrocellulose disc was transferred to BG-11 agar with 50 μg ml kanamycin for selection of pPMQAK1. After 4 weeks, control spots containing cyanobacteria alone will have died back, leaving colonies of successful cyanobacterial transformants behind – these are restreaked directly onto new BG-11 agar plates with 50 μg ml kanamycin and allowed to grow for another 2 weeks. After this period the cyanobacteria are transferred to 10 ml of liquid BG-11 containing 50 μg ml kanamycin using a pipette tip. After a further 2 weeks, this culture can then be grown in 100 ml BG-11 containing 50 μg ml kanamycin in a 250 ml conical flask as with other cyanobacteria.

2.16 RT-PCR

All RNA work was performed in sterile conditions in a designated RNA hood to avoid degradation and/or contamination of samples. RT-PCR was carried out semi-quantitatively in a G-Storm GS1 thermocycler (G-Storm).

2.16.1 RNA extraction

RNA was extracted from cyanobacteria based on a method by Singh et al. 2009. 50 ml cyanobacterial cells were harvested at 4000 *g* for 10 min at 4 °C and immediately resuspended in 1 ml TRI Reagent (Sigma) to stabilize RNA. Samples were then subjected to freeze-thawing in order to break up cell walls, by freezing in liquid nitrogen and thawing in a 70 °C water bath a total of 5 times. 400 μl 1-bromo-3-chloropropane was added and mixed before centrifugation at 16000 *g* for 15 min at 4 °C. The upper aqueous phase was transferred into a new sterile tube and nucleic acids were precipitated by adding 1 ml isopropanol and standing on ice for at least 30 min. RNA was then isolated by using a RNeasy Mini Spin Kit (Qiagen).

2.16.2 Assessment of quality of RNA

Assessment of the quality of total RNA extracted from cyanobacteria was performed using an Agilent 2100 Series Bioanalyzer using the RNA 6000 Nano kit (Aglient). Analysis was performed according to manufacturers protocol using the plant nano RNA assay.

2.16.3 mRNA enrichment from total RNA

In order to improve efficiency of RT-PCR reactions, mRNA was purified from rRNAs that were shown to be present in high concentrations in total RNA extracts. This was performed using MicrobeExpress mRNA isolation kit (Ambion). rRNA was captured by binding to complementary sequences that were in turn bound to magnetic beads and

so could be separated from other RNAs in the mixture with the use of magnets. Purification was performed according to manufacturers protocol.

2.16.4 cDNA synthesis

cDNA was synthesized from RNA using iScript cDNA synthesis kit (Bio Rad). 1 μg RNA was mixed with 1 μl reverse transcriptase and 4 μl reaction buffer in a 20 μl total volume and incubated for 5 min at 25 °C, followed by 30 min at 42 °C and 5 min at 80 °C to inactivate the transcriptase.

2.16.5 Semi-quantitative PCR conditions and method development

Primers were designed using Primer3 web tool (Rozen and Skaletsky, 2000). Primers were designed to amplify candidate sequences that were approximately 400 bp in size and all have a Tm of between 59 °C and 61°C.

Table 2.5. Primers used for RT-PCR. The function of the amplicon-containing gene and forward (F) and reverse (R) primer sequences.

Primers	Function	Sequences
trpA	Tryptophan synthase subunit α (control gene)	F: AAGGCGCTGGAGTGAAAGGTT R: CCCTGGGGTTCCTTCCGCTA
rnpB	RNA subunit of RNase P (control gene)	F: CGGCTTCCCAAAGGCCAAACT R: AGCATGCCACTGGACCCATGG
sigB	RNA polymerase sigma factor rpoD (control gene)	F: CCCGGCTGATGTTGAGATGCTGG R: CGCCCAACAGGGCCGGACTA
accA	acetyl-CoA carboxylase carboxyltransferase α (sll0728)	F: CATCGATGCAGAAAAGTTGG R: TCCTGGGGAGTTAATTGAGC
accB	biotin carboxyl carrier protein (slr0435)	F: TTACGGAACTGCGGGAAT R: TCCACCACAATCTCCATCAC
accC	acetyl-CoA carboxylase biotin carboxylase subunit (sll0053)	F: ATAAATCCACCGCCAAGAAA R: AAGGGACTAGGGGCTTCTTC
accD	acetyl-CoA carboxylase carboxyltransferase β (sll0336)	F: CGGTTTATGGGCGGTAGTAT R; ATCGACAAAACCGTGGTGTA
fabH	3-ketoacyl-ACP synthase III (sll1511)	F: GTTGAACGTAGCAGCCCAAT R: AAGTCGGAGGTGGTCAACTG
fabF1	3-ketoacyl-ACP synthase II subunit 1 (sll1069)	F: AAGGCTTAATGGAGGGTCGT R: CCCTAAGTTGATGGCGGTTA
fabF2	3-ketoacyl-ACP synthase II subunit 2 (slr1332)	F: CTAGTCGGGCTAACCAATGC R: CTAACAAAGCTCCCCCTTCC
fabG1	3-ketoacyl-ACP reductase subunit 1 (sll5079)	F: GAATGTGCTGGGTTTGCTTT R: GAAGACGCCAAGAAGACGAC
fabG2	3-ketoacyl-ACP reductase subunit 2 (slr0886)	F: TGGCTAATGCTGACGAAGTG R: GGCAATGAAACCAGGAGCTA
fabG3	3-ketoacyl-ACP reductase subunit 3 (slr1994)	F: ATGCCGGTATCACCAAAGAC R: CAATTTCCTCCGGTTTACCA
fabZ	3-hydroxyacyl-ACP dehydratase (sll1605)	F: GATTTACTGCCCCACCGTTA R: TTGTCCATCGACCCTAGCTT
fabI	Enoyl-ACP reductase (slr1051)	F: TGAAAAGAAAGTGCGGGAGT R: CAATTCTGCGGCCAGATAAC
cer8	Acyl-ACP synthase* (slr1609)	F: CATAAGGTACGCCAGGCCGCTG R: GGTGTCCTTGGCCCGACCG

*Kaczmarzyk and Fulda, 2010

Reaction Mix (20 μl):

Taq DNA polymerase (New England Biolabs)	0.1 μl
10x Taq buffer	2 μl
dNTPs, 10mM	0.4 μl
forward primer	0.4 μl
reverse primer	0.4 μl
cDNA	0.4 μl
nuclease free water	16.3 μl

PCR Program:

Step	Temperature	Duration	
Initial denaturation	98 °C	30 s	
Denaturation	98 °C	10 s	} *n* cycles*
Annealing	60 °C	30 s	
Extension	72 °C	6 s	
Final extension	72 °C	5 min	

* A range of cycle numbers were tested, it was decided that 25 cycles was sufficient for the final analysis.

PCRs were ran on 1.5 % agarose gels and imaged as described in 2.15.6. Band intensities were measured using ImageJ software and normalized according to the intensity of control gene *trpA*, as this gene had the most consistent expression of the three control genes tested (3.4.2).

2.16.6 Qualitative RT-PCR

For the determination of whether β-oxidation genes in *Acaryochloris* HICR111A were transcribed, a purely qualitative RT-PCR approach was used.

As HICR111A doesn't have a published genome sequence, primers for the amplification of specific sequences found in β-oxidation genes were designed using the genomic sequences of PCC 7425 and MBIC11017, as these are the most closely related organisms according to 16S rRNA sequencing data (Mohr et al. 2010). Protein sequence of interest from MBIC11017 was aligned against its ortholog in PCC 7425 and regions where both sequences were identical for ≥ 6 peptide residues (Fig 2.5, A)

were back-translated into their respective 18mer nucleotide sequences (Fig 2.5, B) using a 6-frame translation tool (http://molbiol.ru/eng/scripts/01_13.html). Candidate primers were analysed using an online oligonucleotide properties calculator (http://www.unc.edu/~cail/biotool/oligo/) to determine they had GC content between 40 % and 60 %, annealing temperatures between 50 °C and 68 °C, have no hairpin secondary structure or self-complement. Primer sequences were searched against both MBIC11017 and PCC 7425 genomes to make sure they only bind to the region that is to be amplified. The primers used and their respective PCR product sizes are listed in table 2.6.

Table 2.6. Primers used for amplification of putative β-oxidation genes from gDNA and cDNA of HICR111A. Respective PCR product sizes listed.

Primer	**Function**	**Sequence**	**Product size**
16SCY	16S rRNA	F: GGCTCAGGATGAACTGGCGG R:ACCTTGTTACGACTTCACCCCAGTC	1.4 Kb
ACS	Acyl-CoA synthetase	F: AATGTGGTCCGTAGTTCCC R: TGCGGCTCAATATTTTCACC	1.2 Kb
ACDH	Acyl-CoA dehydrogenase	F: GAAGAACCTCTAGCCCGGATT R: ATGGCCGTGTAGATATTGGCTAA	0.2 Kb
ECH	Enoyl-CoA hydratase	F: ATGAGCTACACCACGTTC R: TTATTGGATCTCCTGCACA	0.8 Kb
3-HCDH	3-hydroxyacyl-CoA dehydrogenase	F: ATCTCCTGGATCTGCCG R: ACCGAGGGTATTGCCCTT	1.4 Kb
ACT	Acetyl-CoA acyltransferase	F: TATATCGTCAGTAGTGTCCG R: GTTGCTGCTGCCCCATC	0.8 Kb

A.

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MBIC11017    IHNRRHDPMGVPFYNSPLEGHDVivpigqiiggieqaggqWKMIMQTLAAGRGISFPATC  1140
Consensus    + RHDPMGVPF+NSP+ G DV++ + QIIGG+EQAGQGWKM+MQTLAAGRGISFPA+C
PCC 7425     TISTRHDPMGVPFFNSPIAGQDVVISVDQIIGGVEQAGQGWKMLMQTLAAGRGISFPASC  378

MBIC11017    TGVTKLvarvagahavvrKQFGLSIGRFEGVEEPLARIGGFTYMIDAARLYTCGAVDQGE  1320
Consensus    TGV KLVARV GA+A VR+QFGLSIGRFEG+EEPLARIGG TY++DAAR+YTCGAVD GE
PCC 7425     TGVAKLVARVTGAYARVRQQFGLSIGRFEGIEEPLARIGGLTYLLDAARIYTCGAVDGGE  438

MBIC11017    QPAVVSAIAKSQTTELARRVVLDGMDILGGAGICRGPRNLLANIYTAMPIAITVEGANIL  1500
Consensus    +P VVSAIAK   TE+AR++V DGMDILGGAGICRGPRNLLANIYTAMPI ITVEG+NIL
PCC 7425     RPPVVSAIAKYHFTEIARKLVNDGMDILGGAGICRGPRNLLANIYTAMPIPITVEGSNIL  498

MBIC11017    TRSLMIFGQGAIRSHPYIYDEILALEQSDVAAFDQAFWSHLGLIVRNGVragllcltrgr  1680
Consensus    TR++MIFGQG IR HPY+Y E+ AL+  +   FDQ FW HLG +VRN  RA LL LTRGR
PCC 7425     TRTMMIFGQGVIRCHPYLYREVTALQNREAIGFDQTFWQHLGFLVRNSCRALLLGLTRGR  558
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B.

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DNA:gcccatgctgtggtccgcaaacagtttggtttatccattggccgctttgaaggggttgaagaacctctagcccggattgggggа
+1fr·A··H··A··V··V··R··K··Q··F··G··L··S··I··G··R··F··E··G··V··E··E··P··L··A··R··I··G··G·
DNA:ttcacttacatgattgatgcggcccgcctctatacctgtggcgcagtagatcaaggcgaacagcctgcggtggtatccgcaatc
+1fr: ·F··T··Y··M··I··D··A··A··R··L··Y··T··C··G··A··V··D··Q··G··E··Q··P··A··V··V··S··A··
I·
DNA:gcaaaatcccaaaccacagaactagctcgtcgcgtcgtactggatggaatggatattctgggggggagccgggatttgtcgaggg
+1fr: ·A··K··S··Q··T··T··E··L··A··R··R··V··V··L··D··G··M··D··I··L··G··G··A··G··I··C··R··
G·
DNA:ccacgaaacctattagccaatatctacacggccatgccaattgccattaccgtggaggggcaaatattctgacgcgatcgctg
+1fr·P··R··N··L··L··A··N··I··Y··T··A··M··P··I··A··I··T··V··E··G··A··N··I··L··T··R··S··L·
```

Figure 2.5. An example of primer design for amplification of HICR111A acyl-CoA dehydrogenase. **A.** Protein sequence alignments of MBIC11017 and PCC 7425 acyl-CoA dehydrogenase, with conserved regions highlighted in yellow boxes. **B.** Back-translated conserved regions from A used as primer (yellow boxes) to amplify region of DNA, highlighted in blue.

2.16.7 Cyanobacterial growth and feeding conditions

2 separate experiments were carried out to investigate the effects of 1. growth phase (exponential phase vs. stationary phase) and 2. Feeding of a range of fatty acids to PCC 6803.

1. Growth phase

PCC 6803 was harvested as described in 2.16.1 after 4 days at an OD_{730nm} of approximately 6 for the exponential samples and after 9 days at an OD_{730nm} of approximately 10.

2. Feeding of fatty acids

It was determined that the optimum way to feed fatty acids was by dissolving in ethanol at a final concentration of 1% v/v (see 3.1.3) Acetate, decanoic acid, palmitic acid and stearic acid were fed to PCC 6803 at an OD_{730nm} of 6 for 24 h at a working concentration of 0.3 mM. In addition, controls of ethanol (solvent for decanoic acid, palmitic acid and stearic acid) and water (solvent for acetate) were carried out.

3. Results

3.1 Cyanobacteria lack β-oxidation

3.1.1 Introduction

The following results demonstrate that there is no β-oxidation pathway in cyanobacterial strains PCC 6803, PCC 7120 and PCC 7937. This conclusion is supported by the following observations; 1. Making use of bioinformatic tools in order to show that possible candidate protein sequences for enzymes with apparent β-oxidation activity in fact have a closer orthology with other enzymes not involved in β-oxidation; 2. Experimentally by development of a technique for the detection of the most abundant long-chain acyl-coenzyme A thioesters, the substrates of β-oxidation, by using triple-quadrupole liquid-chromatography mass-spectrometry (LCMS-QQQ); 3. Assaying for the activity of the rate limiting enzymes of β-oxidation, acyl-CoA dehydrogenase and acyl-CoA oxidase; 4. A pulse-chase experiment, feeding ^{14}C labeled fatty acid to cyanobacteria and detecting resulting metabolites incorporating radioactivity originating from the fatty acids.

3.1.2 Bioinformatic analyses

40 strains of cyanobacteria were assessed using the KEGG Pathway tool in order to determine if they had gene ontologies computationally assigned to them for enzymes involved in the β-oxidation pathway (Table 3.1). Of the 40 assessed, *Cyanothece* PCC 7425 had all genes present. *Acaryochloris marina* MBIC 11017 was the only other strain to have more than one of the genes required for β-oxidation present, having all except acetyl-CoA acetyltransferase. *Synechocystis* PCC 6803, *Synechococcus* CC9605, *Microcystis aeruginosa*, *Cyanothece* PCC 7424, *Cyanothece* PCC 7822, *Gleobacter violaceus*, *Nostoc* PCC 7120, *Nostoc punctiforme* PCC 73102 and *Anabaena variabilis* PCC 7937 all had one gene present. The next step was to systematically determine whether ontologies for each of these enzymes were possible misannotations, and to search for orthologous sequences in other cyanobacterial strains.

Table 3.1. Computational annotation of the 5 major β-oxidation enzymes in 40 cyanobacterial strains according to the KEGG database. Green boxes indicate computational annotation assigned.

Strain	**Acyl-CoA dehydrogenase (1.3.99.-)**	**Enoyl-CoA hydratase (4.2.1.-)**	**3-hydroxyacyl-CoA dehydrogenase (1.1.1.35)**	**Acetyl-CoA acyltranferase (2.3.1.16)**	**Acetyl-CoA acetyltranferase (2.3.1.9)**
Synechocystis PCC 6803					
Synechococcus sp. WH8102					
S. elongatus PCC6301					
S. elongatus PCC7942					
Synechococcus sp. CC9605					
Synechococcus sp. CC9902					
Synechococcus sp. CC9311					
Synechococcus sp. RCC307					
Synechococcus sp. WH7803					
Synechococcus sp. PCC7002					
Yellowstone A-Prime					
Yellowstone B-Prime					
Thermosynechococcus elongatus					
Microcystis aeruginosa					
Cyanothece sp. ATCC 51142					
Cyanothece sp. PCC 8801					
Cyanothece sp. PCC 7424					
Cyanothece sp. PCC 7425					
Cyanothece sp. PCC 8802					
Cyanothece sp. PCC 7822					
Cyanobacterium UCYN-A					
Gloeobacter violaceus PCC 7421					
Nostoc sp. PCC7120					
Nostoc punctiforme PCC 73102					
Anabaena variabilis PCC 7937					
A. azollae 0708					
Prochlorococcus marinus SS120					
P. marinus MED4					
P. marinus MIT 9313					
P. marinus NATL2A					
P. marinus MIT9312					
P. marinus AS9601					
P. marinus MIT 9515					
P. marinus MIT 9303					
P. marinus MIT 9301					
P. marinus MIT 9215					
P. marinus MIT 9211					
P. marinus NATL1A					
Trichodesmium erythraeum					
Acaryochloris marina MBIC 11017					

1. Acyl-CoA dehydrogenase

This enzyme is present according to KEGG in PCC 7425 and MBIC 11017 (EC:1.3.99.-) (Table 3.1), so these two sequences were searched against 38 other fully sequenced cyanobacterial genomes using BLAST. Six significant hits were generated against both sequences, 2 of which are hypothetical (Table 3.2). All of these sequences had strong BLAST alignments with non-ribosomal peptide synthetases of other microbes such as those in the genus of pathogenic bacteria *Burkholderia*, and all have acyl-CoA dehydrogenase N- middle- and C-terminal domains as well as an AMP-binding domain and phosphopantetheine attachment site (Fig. 3.1), the only exception to this is the AMP-dependent synthetase and ligase in *N. punctiforme*, which has the EC number 1.3.99.2 and is a predicted butyryl-CoA dehydrogenase, involved in butanoate metabolism (Fig. 3.1, PCC73102-A).

Table 3.2. BLAST hits returned against *Cyanothece* PCC 7425 and *Acaryochloris marina* MBIC 11017 acyl-CoA dehydrogenases.

Organism	**Entry**	**Score**	**E-value**
Crocosphaera watsonii	AMP-dependent synthetase and ligase:Acyl-CoA dehydrogenase	90.1	2e-17
Cyanothece ATCC 51142	AMP-dependent synthetase and ligase, acyl-CoA synthase	81.6	7e-15
Nostoc punctiforme PCC 73102 *(A)*	Acyl-CoA dehydrogenase domain-containing protein	79.3	4e-14
Nostoc PCC 7120	Hypothetical protein alr2678	66.6	3e-10
Nostoc punctiforme PCC 73102 *(B)*	AMP-dependent synthetase and ligase	64.3	1e-09
Nodularia spumigena CCY 9414	Hypothetical protein N9414_20870	63.2	3e-09

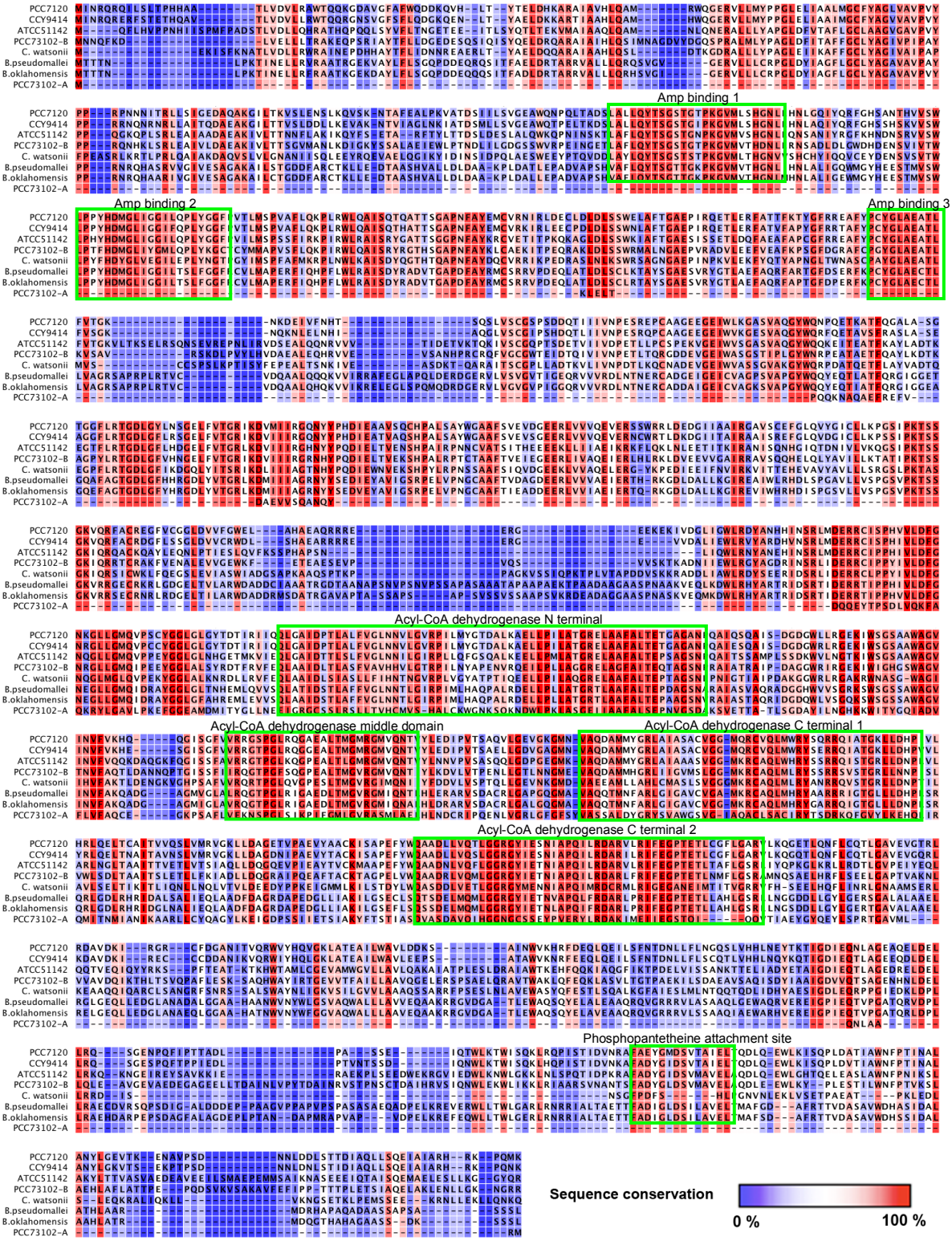


Figure 3.1. Alignment of 6 BLAST hits from Table 3.3 and 2 non-ribosomal peptide synthetases from *Burkholderia* species. Conserved domains highlighted in green boxes. Red colours indicate high conservation between the sequences listed and blue colours indicate a lack of conservation.

2. Enoyl-CoA hydratase

Enoyl-CoA hydratase (EC 4.2.1.17) has been predicted by sequence similarity in 4 cyanobacterial species on the KEGG database: MBIC 11017, PCC 7425, PCC 73102 and PCC 7937 (Table 3.1). These sequences from the above strains were BLAST searched against other cyanobacteria. No sequences producing significant alignments against other cyanobacterial genomes were found when searching using sequences from species MBIC 11017, PCC 7425 and PCC 73102.

Against PCC 7937, 2 significant results were returned from PCC 7120; one a hypothetical protein, *alr4455* with a score of 509 and E-value of 3e-144, and the other in *Gloeobacter violaceus* PCC 7421, 6-oxocamphor hydrolase with a score of 428 and E-value of 6e-120 (Table 3.3). 6-oxocamphor hydrolase is a member of the crotonase superfamily to which enoyl-CoA hydratase belongs (Holden, 2001). Entry *alr4455* is a β-diketone hydrolase (Bennett et al. 2007) and interestingly does not use –CoA thioesters as a substrate, unlike other types of this enzyme. MBIC 11017, PCC 7425 and PCC 73102 sequences searched here have since been found to have an ortholog in PCC 6803 that has been identified and experimentally characterized as a 1,4-dihydroxy-2-naphthoyl-CoA synthase (Song and Guo, 2012). The sequences of 1,4-dihydroxy-2-naphthoyl-CoA synthases from MBIC 11017, PCC 7425 and PCC 73102 (Fig. 3.2, A) and of hydrolases from PCC 7421, PCC 7937 and PCC 7120 (Fig. 3.2, B) all show high conservation. All sequences have the enoyl-CoA hydratase domain according to Pfam analysis.

Table 3.3. 2 significant BLAST hits returned against *A. variabilis* PCC 7937 enoyl-CoA hydratase.

Organism	**Entry**	**Score**	**E-value**
Nostoc PCC 7120	Hypothetical protein alr 4455	509	3e-194
Gleobacter violaceus	6-oxocamphor hydrolase	428	6e-120

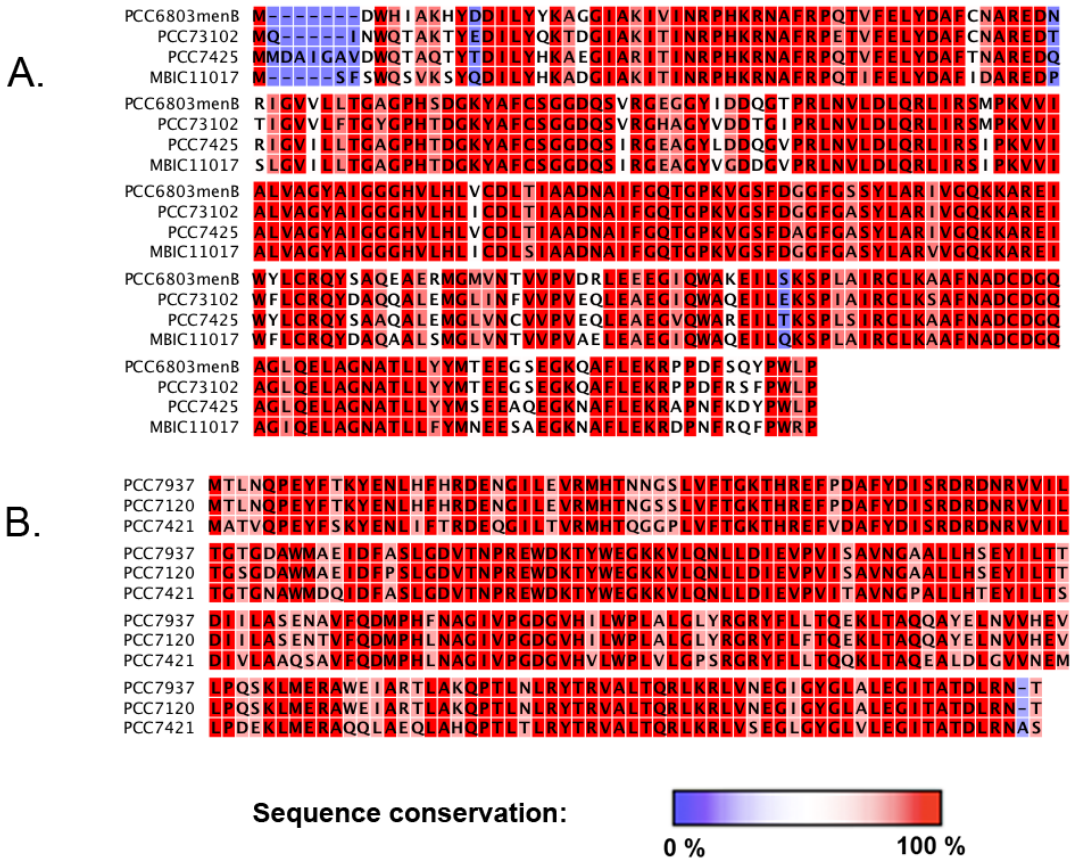


Figure 3.2. Alignments with cyanobacterial enoyl-CoA hydratases. **A.** Alignment of a 1,4-dihydroxy-2-naphthoyl-CoA synthase from PCC 6803 (PCC6803menB) with cyanobacterial sequences identified on KEGG as enoyl-CoA hydratases from PCC 73102, PCC 7425 and MBIC 11017. **B.** Alignment of hydrolases from PCC 7937, PCC 7120 and PCC 7421. Red colours indicate high conservation between the sequences listed and blue colours indicate a lack of conservation.

3. 3-hydroxylacyl-CoA dehydrogenase

3-hydroxylacyl-CoA dehydrogenase (EC 1.1.1.35) is present in PCC 7425 and MBIC 11017 according to KEGG (Table 3.2). The only sequence showing significant alignment to these two sequences is a 3-hydroxybutyryl-CoA dehydrogenase (EC 1.1.1.157) from PCC 73102, with scores of 85.5 and 109 and E values of 3e-17 and 3e-25 respectively. 3-hydroxybutyryl-CoA dehydrogenase is involved in phenylalanine metabolism, benzoate metabolism and butanoate metabolism, however other enzymes in these pathways are not present according to KEGG so this enzyme is likely not to be involved in these pathways either. This sequence aligns with a diverse array of 3-hydroxybutanoyl-CoAs from other bacteria such as Firmicutes (*Bacillus mycoides*), Chloroflexi (*Herpetosiphon aurantiacus*) and δ-proteobateria (*Sorangium cellulosum*, *Haliangium ochraceum*) (Fig. 3.3,C). 3-hydroxylacyl-CoA dehydrogenase from PCC 7425 and MBIC 11017 shows high conservation with orthologs from Bacteroidetes, but lower conservation with more distantly related bacteria, such as γ-proteobacteria (Fig 3.3, A and B). Another observation is that 3-hydroxybutyryl-CoA dehydrogenases are also much shorter in sequence length than 3-hydroxyacyl-CoA dehydrogenases, being 290 ± 13 residues in length versus 761 ± 45 residues.

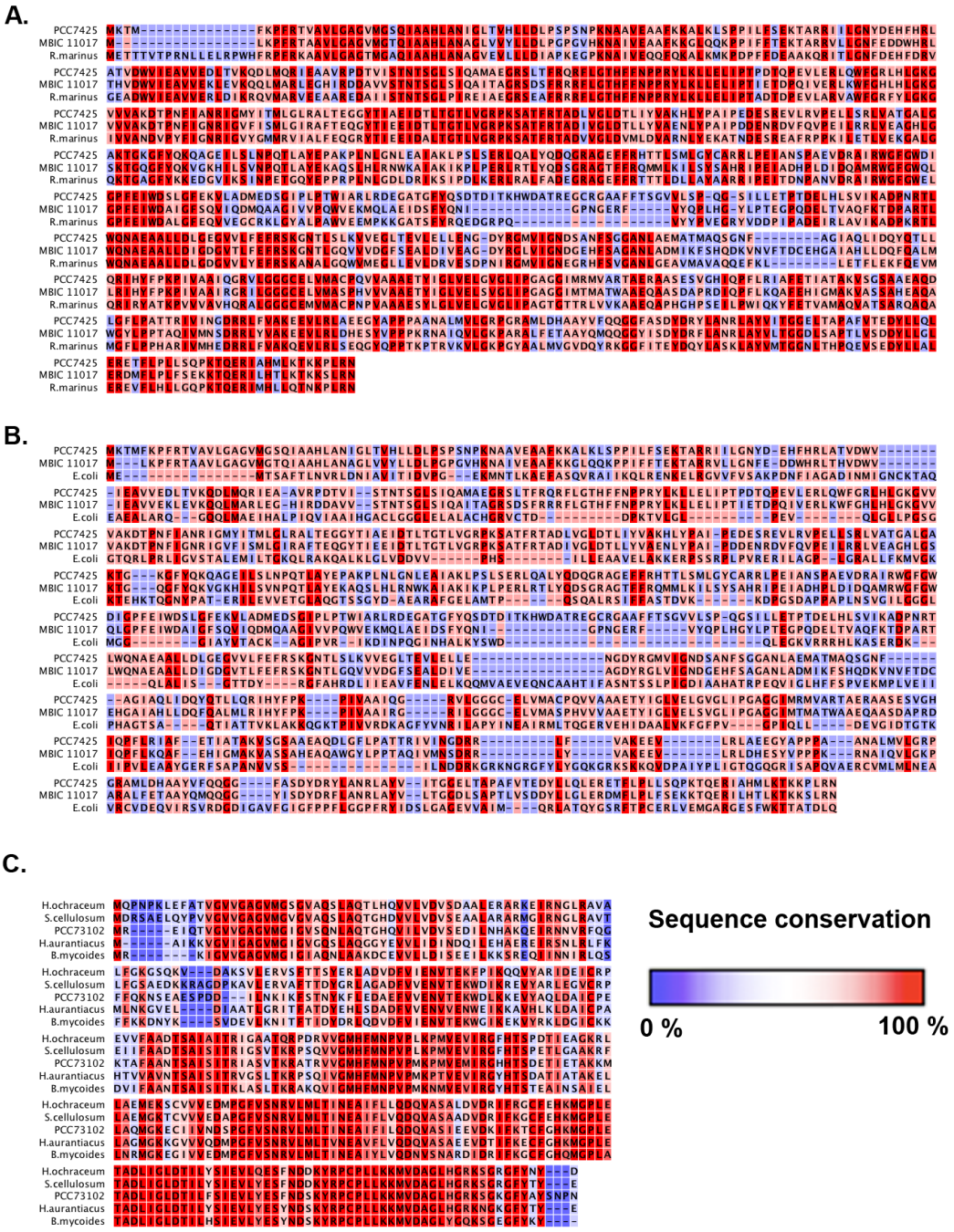


Figure 3.3. Alignments of 3-hydroxyacyl-CoA dehydrogenases. **A.** Alignment of 3-hydroxyacyl-CoA dehydrogenase in PCC 7425, MBIC 11017 and the Bacteroidete *R.marinus* showing high sequence conservation. **B.** Alignment of 3-hydroxyacyl-CoA dehydrogenase in PCC 7425, MBIC 11017 and *E.coli* showing low sequence conservation. **C.** Alignment of PCC 73102 3-hydroxybutyryl-CoA dehydrogenase with a range of bacterial orthologs. Red colours indicate conservation and blue colours indicate a lack of conservation.

4. Acetyl-CoA acyltransferase and Acetyl-CoA acetyltransferase

Acetyl-CoA acyltransferase (EC: 2.3.1.16) is present in MBIC 11017 and PCC 7425 according to KEGG (Table 3.2), so these two sequences were BLAST searched against 38 other fully sequenced cyanobacterial sequences. 4 significant hits were generated against both sequences (Table 3.4), 3 of these were acetyl-CoA acetyltransferases (EC: 2.3.1.9), as opposed to acyltransferases, already present according to KEGG (Table 3.2).

Interestingly, a hit was returned from *M. aeruginosa*, annotated as a polyhydroxyalkanoate-specific β-ketothiolase, involved in the biosynthesis of polyhydroxyalkanoates (PHAs). PHA biosynthesis has been reported in PCC 6803 (Hein et al. 1998), the genes for which being encoded by *slr1829* and *slr1830*. The initial starting substrate of PHA biosynthesis is acetyl-CoA, of which 2 molecules are condensed into acetoacetyl-CoA by the polyhydroxyalkanoate-specific β-ketothiolase (encoded by *phaA*), this is then converted into 3-hydroxyacyl-CoA by acetoacetyl-CoA reductase (*phaB*), before being polymerised into PHA by PHA synthase (*phaC*, *phaE*) additionally, PHAs can also be produced from acyl-ACPs or acyl-CoAs from fatty acid biosynthesis or β-oxidation respectively (Steinbuchel and Lutke-Eversloh, 2003). Sequences from organisms that have characterized PHA biosynthetic pathways were used to search for the presence of other PHA enzymes in cyanobacteria using BLAST. The organisms used were PCC 6803, *Ralstonia eutropha* H16 (Oeding and Schlegal, 1973) and *Allochromatium vinosum* (Yuan et al. 2001). Hits from cyanobacteria in table 3.5 all had strong alignments with PHB genes from the above organisms as can be seen from their high bit scores. From the data obtained it also seems that the cyanobacterial strains possess a class III PHA synthase, as the higher bit scores are obtained against the class III PHA synthase of *A. vinosum*. Therefore it appears that these hits returned as acetyl-CoA acetyl transferases may be involved in PHA biosynthesis and not in β-oxidation.

Table 3.4. BLAST hits returned against *Cyanothece* PCC 7425 and *Acaryochloris marina* MBIC 11017 acetyl-CoA acyltransferase.

Organism	**Entry**	**Score**	**E-value**
Cyanothece PCC 7822	Acetyl-CoA acetyltransferase	217	5e-65
Cyanothece PCC 7424	Acetyl-CoA acetyltransferase	206	5e-61
Synechocystis PCC 6803	Acetyl-CoA acetyltransferase	196	6e-57
Microcystis aeruginosa	PHA-specific β-ketothiolase	200	2e-58

Table 3.5. Significant bit scores obtained when protein sequnces of enzymes involved in PHA biosynthesis were BLAST searched against 40 cyanobacterial genomes. PhaC and PhaE are PHA synthases. PhaB is an acetoacetyl-CoA reductase,

Organism	**Bit score**					
	A. vinosum		***R. eutropha***		**PCC 6803**	
	PhaC Class III	**PhaE**	**PhaC Class I**	**PhaB**	**PhaE Slr1829**	**PhaC Slr1830**
M. aeruginosa	398	62.4	81.3	179	246	575
PCC 7822	397	63.5	64.3	177	258	556
PCC 7425	396	56.6	68.2	177	270	552
PCC 7424	387	53.5	68.2	190	258	565
PCC 6803	375	47.4	69.3	152	607	772

3.1.3 Acyl-CoAs cannot be detected in cyanobacterial extracts

Method development

Standards of 16:0-CoA, 16:1-CoA and 18:0-CoA were used for the development of a reliable protocol for the detection of these compounds using LC-QQQ-MS. These chain length acyl-CoAs were chosen, as cyanobacteria are known to possess corresponding chain length fatty acids in the highest abundance in cell membranes (Hitchcock and Nichols, 1971). MS2 scans were performed in both positive and negative polarities, signal from standards was found to be approximetely 13-fold more sensitive using positive polarity compared to negative polarity (Fig. 3.4), so the ESI was set to positive polarity for these assays.

The QQQ was set to scan between 800 and 1200 m/z for each standard and found major peaks at 1004.5, 1006.5 and 1034.5 for 16:1-CoA, 16:0-CoA and 18:0-CoA respectively (Fig. 3.5), with lower abundance corresponding adducts of sodium and potassium, which are +22 and +38 m/z heavier.

These masses were subjected to product ion scans by fragmentation in the collision chamber with nitrogen gas, with collision energies between 10 V and 30 V. Major product ions at 497.4, 499.5 and 527.3 m/z for 16:1-CoA, 16:0-CoA and 18:0-CoA respectively were detected (Fig. 3.6). MRMs using these precursor and product masses were tested on standards (Fig. 3.7).

From these transitions obtained, it was possible to determine transitions of other chain-length acyl-CoAs by simply adding or subtracting the mass of a $–CH_2–$ (Table 3.6), it was also possible to determine transitions for acyl-CoAs with functional groups such as –OH groups or C=C bonds. However, it was not possible to reliably quantify the concentration of acyl-CoAs with calculated MRMs as different chain length and functional groups affect the ionisation efficiency of the compound, as can be seen with

the different peak areas in Fig. 3.5, A, where all for all 3 standards a total of 150 pmol was injected.

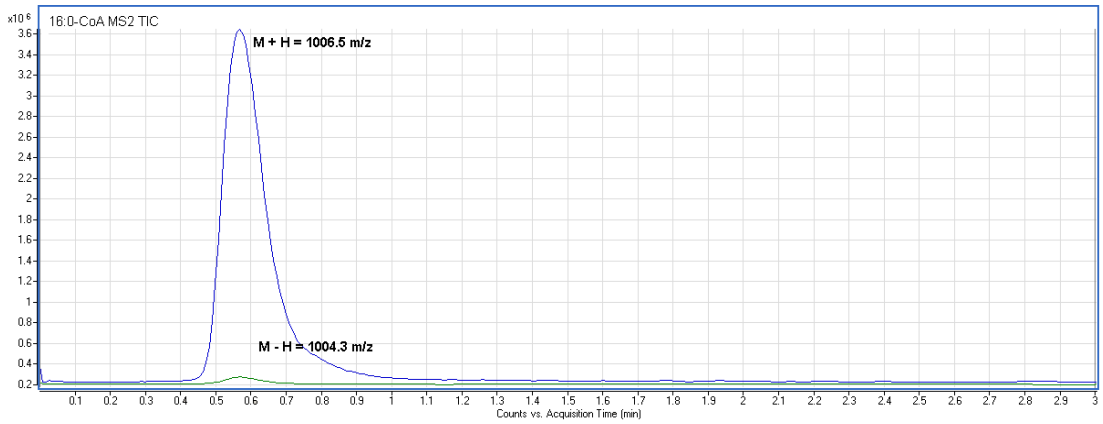


Figure 3.4. Total ion chromatograms (TICs) of MS2 scans of 16:0-CoA standards running in positive polarity (blue trace) giving a M+H m/z = 1006.5; and negative polarity (green trace) giving a M-H m/z = 1004.3. Abundance of M+H is $3.6x10^6$ vs. $2.9x10^5$ for M-H. Integrated peak area for M+H = 30,245,803; and M-H = 400,742.

The detection limit of acyl-CoAs for the instrument assessed using standards of 18:0-CoA and was determined to be 6 pmol where the smallest integratable peak could be observed (Fig. 3.8). Peak area was determined to be linear between of 20 pmol and 1 nmol 18:0-CoA.

As a positive control, n=3 samples of *E. coli* strain DH5α were grown overnight, extracted and enriched by SPE as described in materials and methods, pooled and subjected to the MRM method as described. 20 different acyl-CoAs could be detected, both saturated (Fig. 3.9) and those with functional groups (Fig. 3.10). Relative quantification of 16:1-CoA, 16:0-CoA and 18:0-CoA showed that they were found to be at levels equivalent to those of fatty acids that have been previously observed in *E. coli* (Raetz, 1978), except for in the case of stearoyl-CoA, which is present at a much higher abundance – 7 to 30% for 18:0-CoA vs. 0 to 1% for the corresponding fatty acid (Table 3.8).

The only acyl-CoAs detectable in PCC 6803, PCC 7120 and PCC 7937 were acetyl-CoA and malonyl-CoA at very low abundance (Fig. 3.11). In order to show that other components of the cyanobacterial extracts were not inhibiting ionisation or detection of acyl-CoAs, 150 pmol 18:0-CoA was spiked into the extract and sample was re-injected (Fig. 3.12), an average recovery of 102.9% of internal standard was observed in the cyanobacterial extracts compared to standard alone.

Table 3.6. List of different acyl-CoAs that the LC-QQQ-MS/MS was set up to detect and their respective transitions.

Compound	Precursor m/z (M+H)	Product m/z (M+H)
24:0-CoA	1118.5	611.4
22:0-CoA	1090.5	583.4
22:6-CoA	1076.5	569.4
22:1-CoA	1088.5	581.4
20:0-CoA	1062.5	555.4
20:5-CoA	1052.5	545.4
20:3-CoA	1056.5	549.4
20:1-CoA	1060.5	553.4
18:0-CoA	1034.5	527.3
18:4-CoA	1026.5	519.4
18:3-CoA	1028.5	521.4
18:2-CoA	1030.5	523.4
18:1-CoA	1032.5	525.4
16:0-CoA	1006.5	499.5
16:2-CoA	1002.5	495.4
16:1-CoA	1004.5	497.4
14:0-CoA	978.5	471.4
β-hydroxy-14:0-CoA	994.5	487.4
12:0-CoA	950.5	443.4
10:0-CoA	922.5	415.4
8:0-CoA	894.5	387.4
6:0-CoA	866.5	359.4
4:0-CoA	838.5	331.4
Malonyl-CoA	854.5	347.4
Acetyl-CoA	810.5	303.4

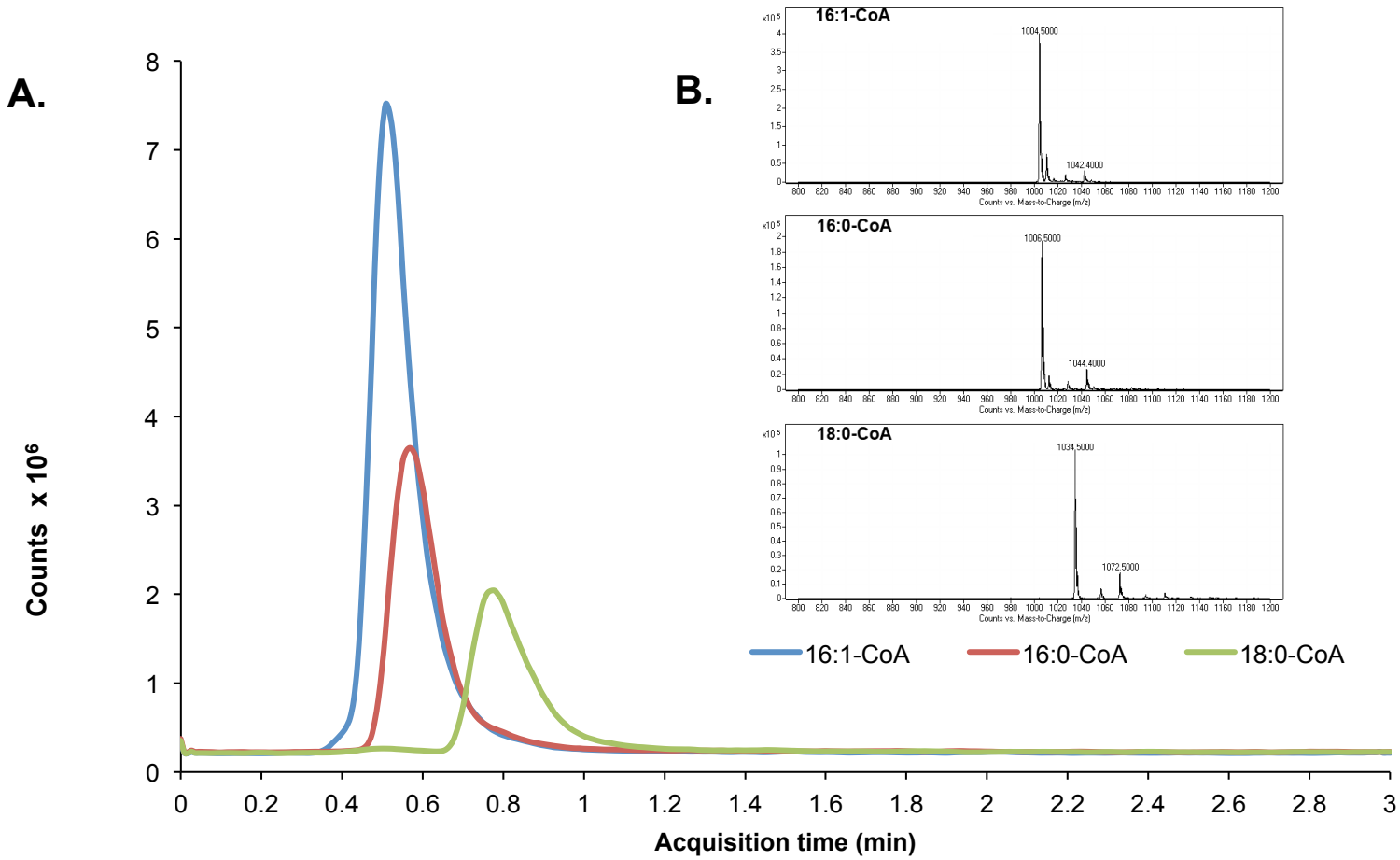


Figure 3.5. (**A**) Total ion chromatograms of acyl-CoA standards 16:1-CoA, 16:0-CoA and 18:0-CoA and (**B**) extracted mass spectra, with corresponding major peaks at 1004.5, 1006.5 and 1034.5 m/z.

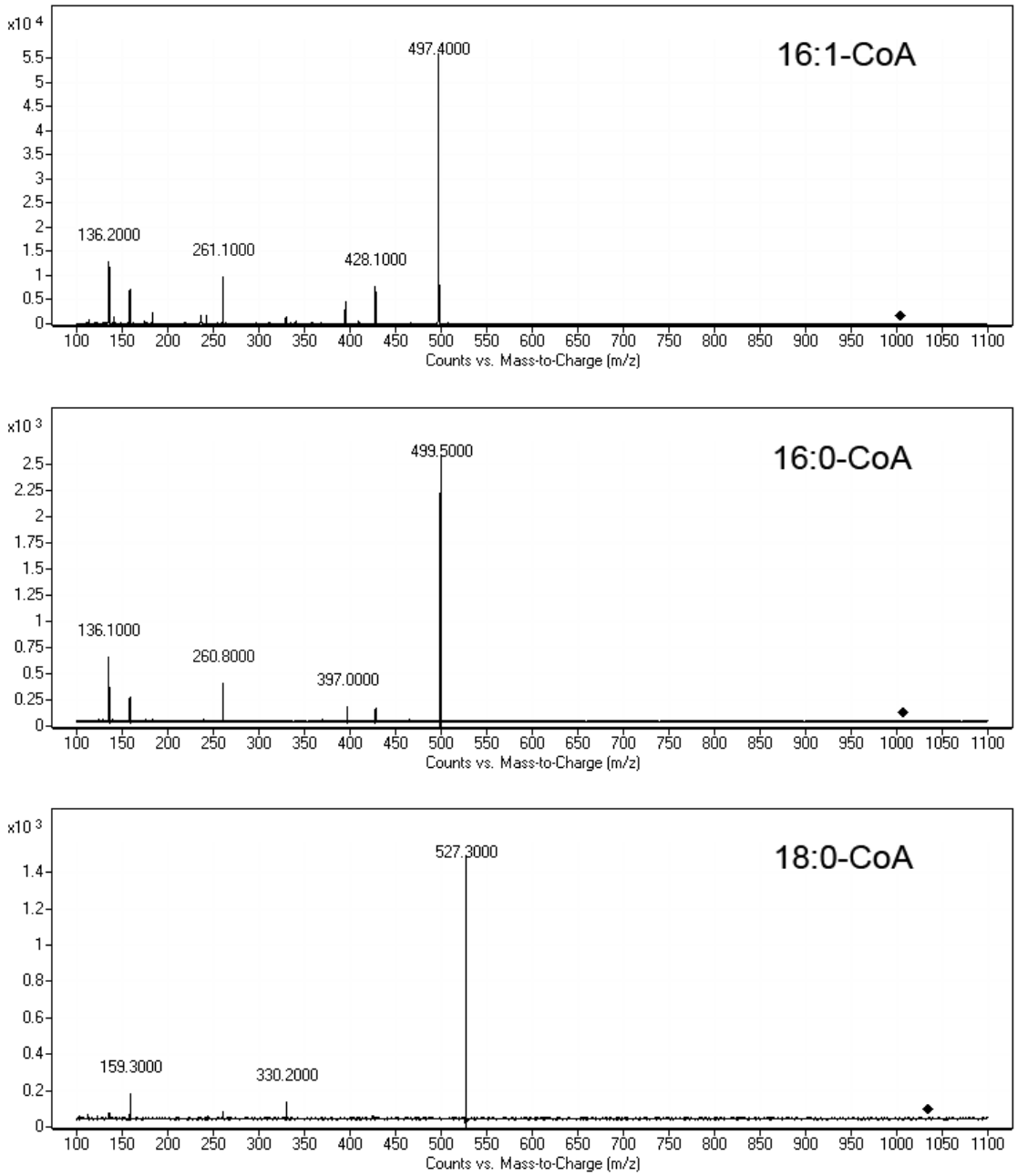


Figure 3.6. Mass spectra of product ions from 16:1-CoA, 16:0-CoA and 16:1-CoA, generated from fragmentation in the collision chamber of the LC-QQQ-MS/MS. Precursor ion is indicated by diamond. Acyl-chain length dependent major product ions at 497.4, 499.5 and 527.3 m/z.

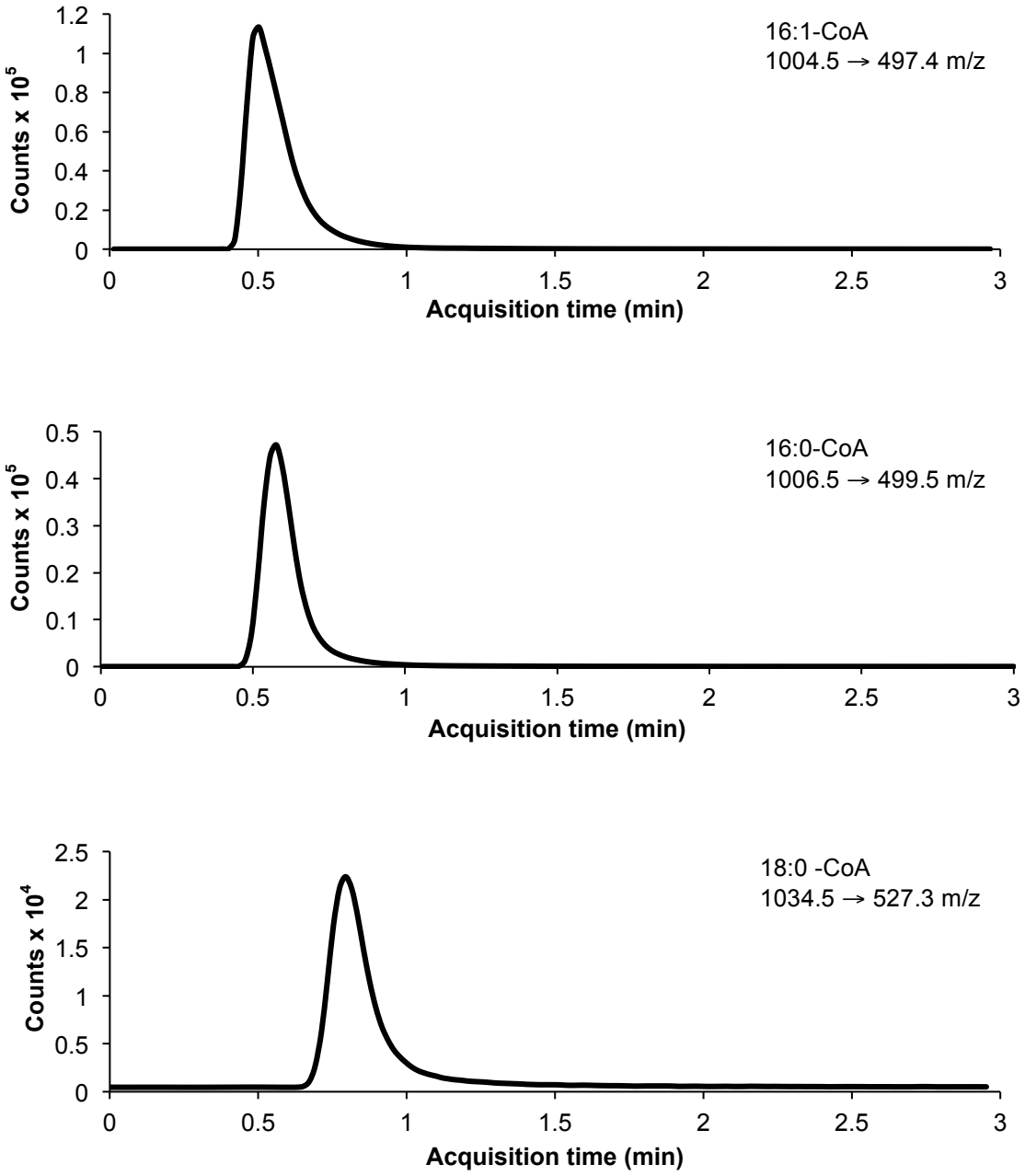


Figure 3.7. Chromatograms from multiple reaction monitors performed on standards of 16:1-CoA, 16:0-CoA and 18:0-CoA. Their respective transitions resulting from CID of precursor ion to acyl-chain length dependent product ions are shown. Transitions were 1004.5 to 497.4 m/z for 16:1-CoA, 1006.5 to 499.5 m/z for 16:0-CoA and 1034.5 to 527.3 m/z for 18:0-CoA.

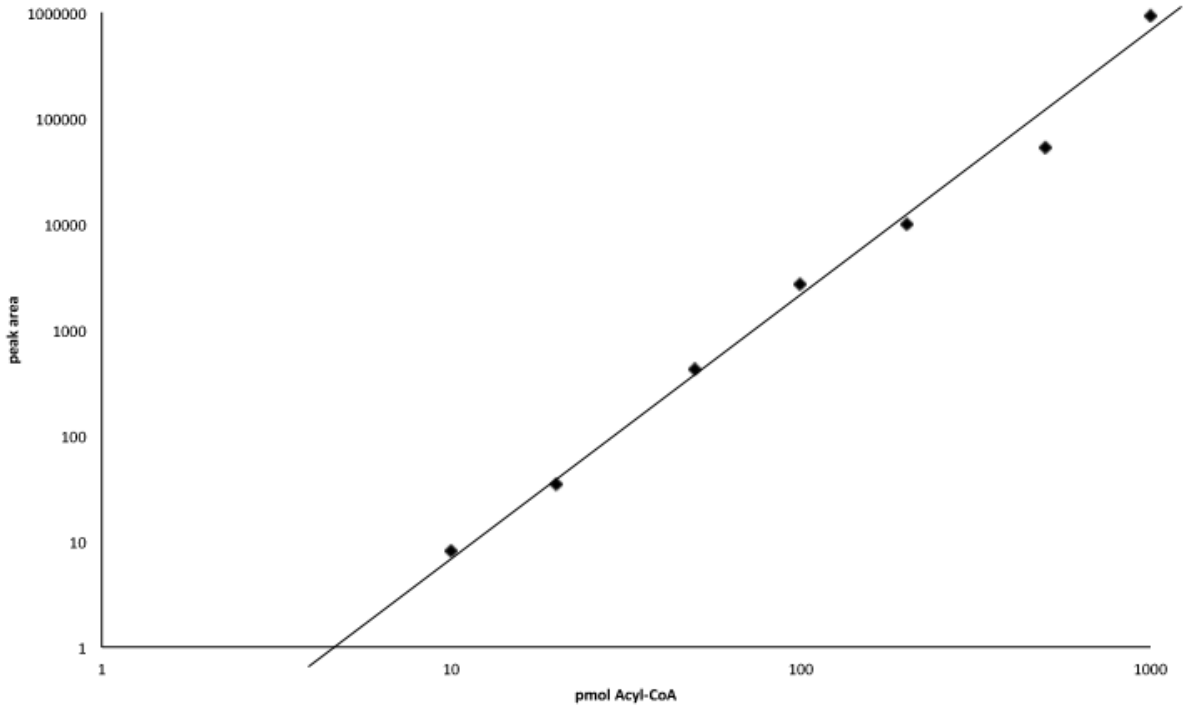


Figure 3.8. LCMS-QQQ standard curve of 18:0-CoA SRM with transition 1034.5 m/z to 527.3 m/z, between 1 pmol and 1200 pmol of 18:0-CoA. Quantification was performed by integrating peak areas on total ion chromatograms using MassHunter Qualitiative Analysis software. Detection limit was determined to be at 6 pmol. At amounts above 1200 pmol the instrument response was no longer linear.

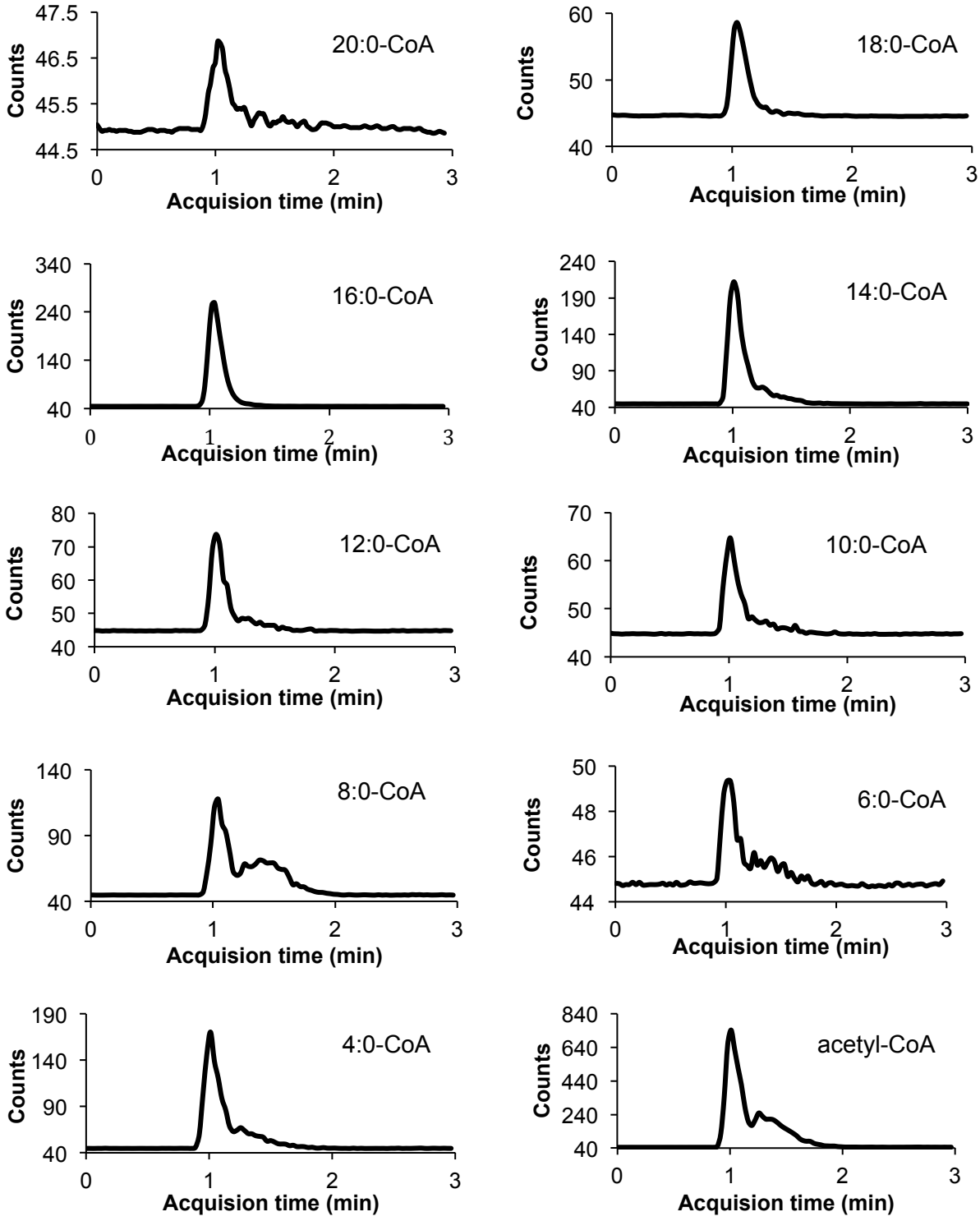


Figure 3.9. Extracted MRM chromatograms of fully saturated acyl-CoAs in *E. coli* that contain no functional groups on the acyl chain.

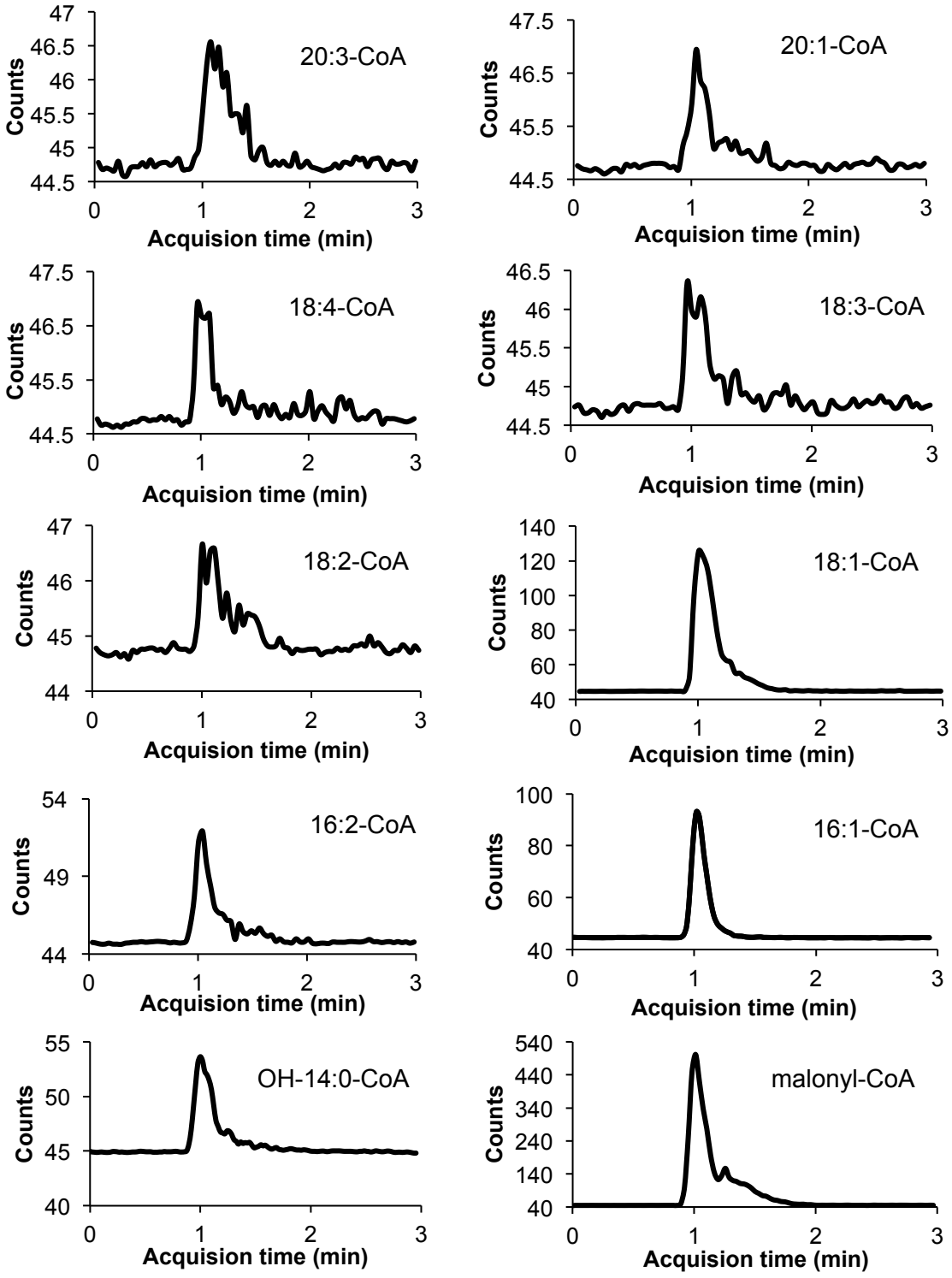
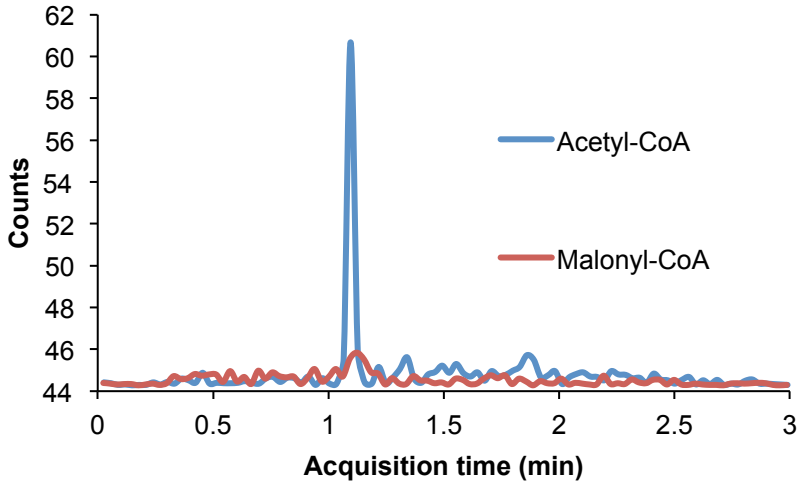


Figure 3.10. Extracted MRM chromatograms of acyl-CoAs in *E. coli* that contain C=C double bonds and –OH groups.

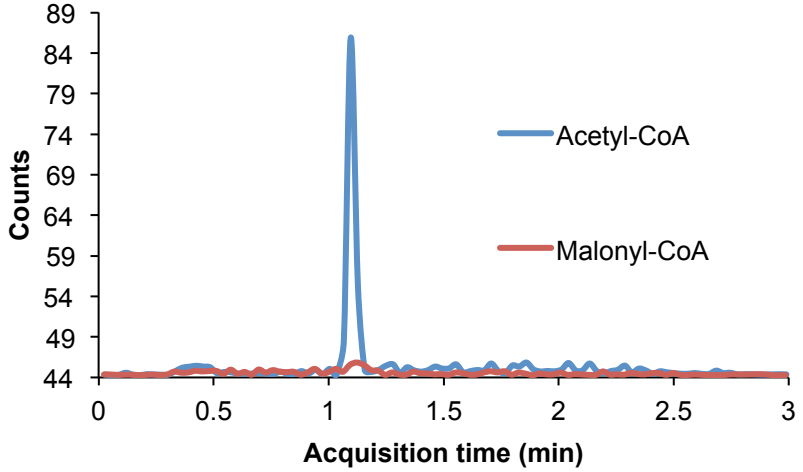
Table 3.7. Composition of 16:1-CoA, 16:0-CoA and 18:0-CoA in *E. coli* expressed as a percentage of the three acyl CoAs detected, based on peak area. n = 3.

Acyl-CoA	**Fraction of total (%)**
16:1-CoA	51.5 ± 17.5
16:0-CoA	29.5 ± 17.5
18:0-CoA	18.5 ± 11.5

PCC 6803



PCC 7120



PCC 7937

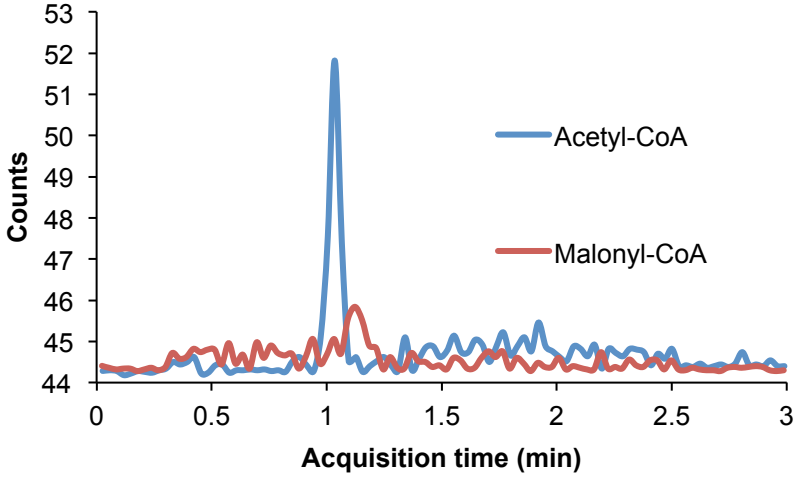


Figure 3.11. Extracted MRM chromatograms of acetyl-CoA and malonyl-CoA in 3 tested cyanobacterial strains.

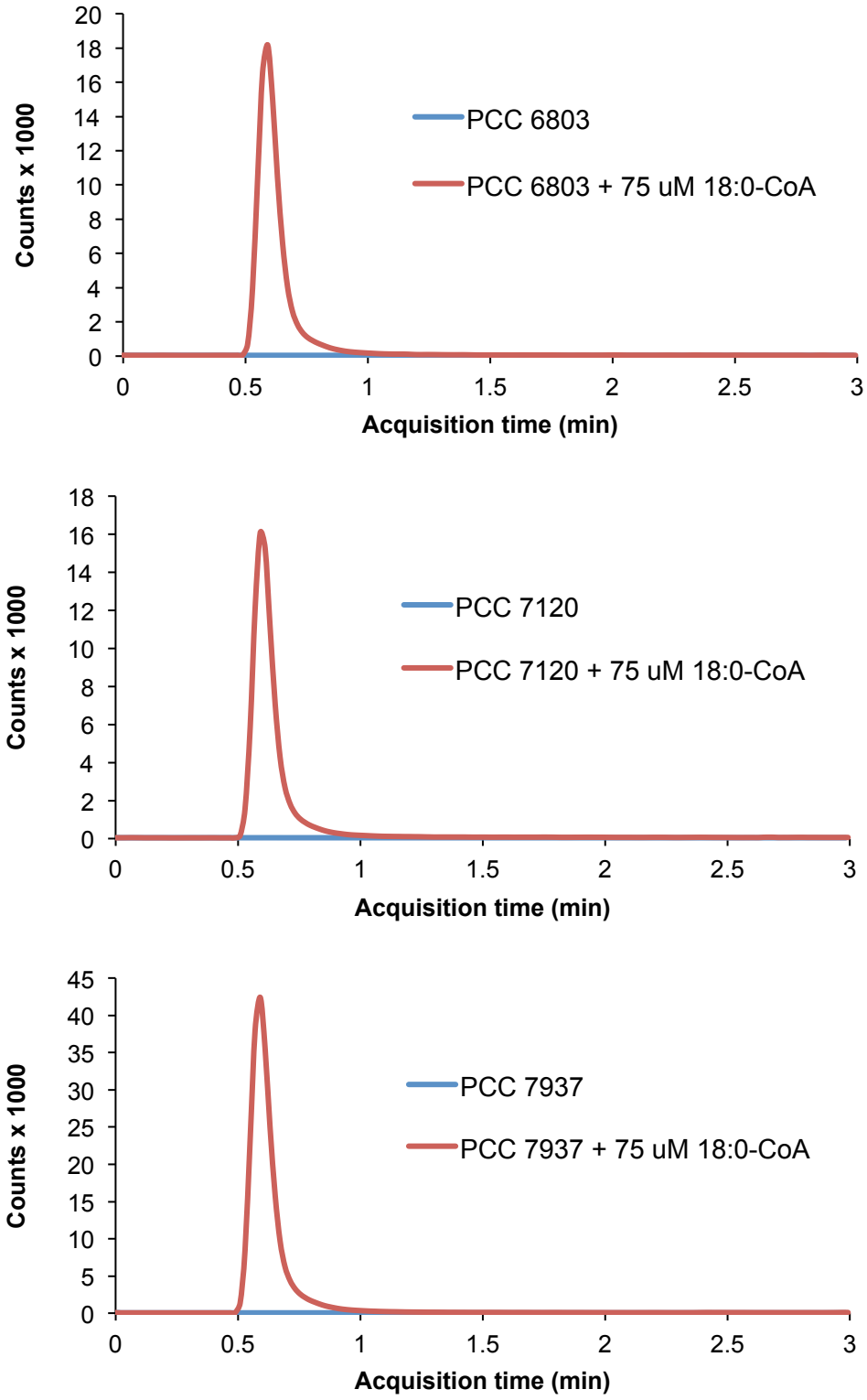


Figure 3.12. Extracted MRMs for 18:0-CoA from extracts of PCC 6803, PCC 7120 and PCC 7937 alone and with 150 pmol of 18:0-CoA spiked into the same extract.

3.1.4 No activity for acyl-CoA dehydrogenase or acyl-CoA oxidase can be detected in cyanobacteria

Acyl-CoA dehydrogenase

This enzyme was assayed according to a method described by Dommes and Kanau, 1979. Acyl-CoA dehydrogenase removes two hydrogen atoms from carbon atoms 2 and 3 on the acyl chain to create a carbon-carbon double bond and transfers the hydrogen atoms to the enzymes FAD prosthetic group, reducing it to $FADH_2$, it is then reoxidised back to FAD by components of the electron transport chain. In this assay, electrons from $FADH_2$ are transferred instead to PMS, which subsequently reduces INT, which gives a colour change from yellow to red (Fig. 3.13) that can be followed spectrophotometrically at 492 nm.

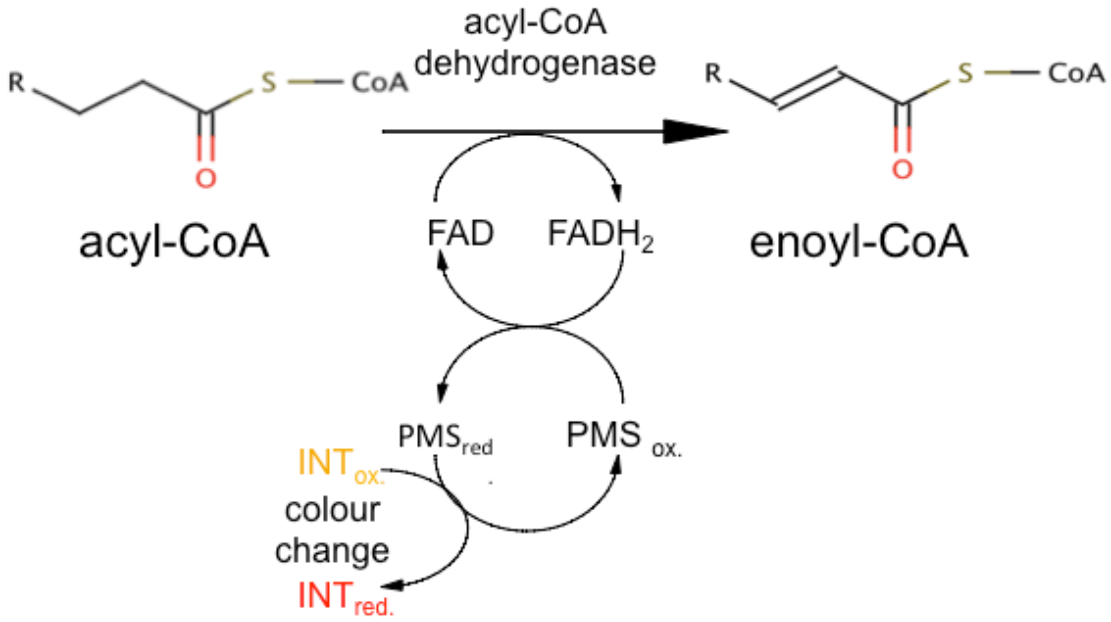


Figure 3.13. Principle of acyl-CoA dehydrogenase assay method.

As a positive control, crude extracts of *E coli* strain DH5α were tested for acyl-CoA dehydrogenase activity, as this strain is known to have a complete β-oxidation pathway (Overath et al.1969). Average initial activity of *E. coli* acyl-CoA dehydrogenase was

found to be 0.522 nmol INT (mg protein^{-1}) min^{-1} (Fig. 3.14). Varying substrate concentrations were assayed, and a double reciprocal graph was plotted, which confirmed that this enzyme follows Michaelis-Menten reaction kinetics, K_M was determined by extrapolating the line to where $1/V$ =0, at which value 1/[S] = -1.54. Therefore K_m = -1/-1.54 = 0.65 mM (Fig. 3.15). k_{cat} could not be reliably calculated as the enzyme was part of a crude protein mixture.

Crude protein extracts of 3 cyanobacterial strains were assayed by the same method (Fig. 3.14) however, negligible activity could be found above baseline rate of reduction of INT.

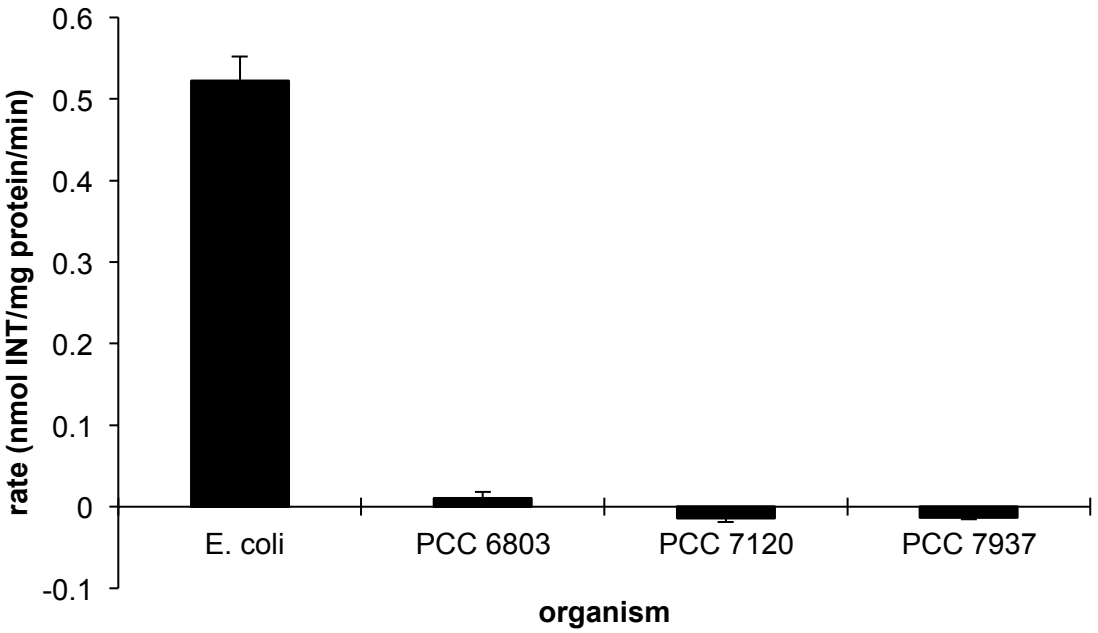


Figure 3.14. Comparison of initial rates of acyl-CoA dehydrogeanse activity as quantified by nmol of INT reduced per mg of protein in crude extract per minute in *E. coli*, PCC 6803, PCC 7120 and PCC 7937. Error bars show S.E. n = 6.

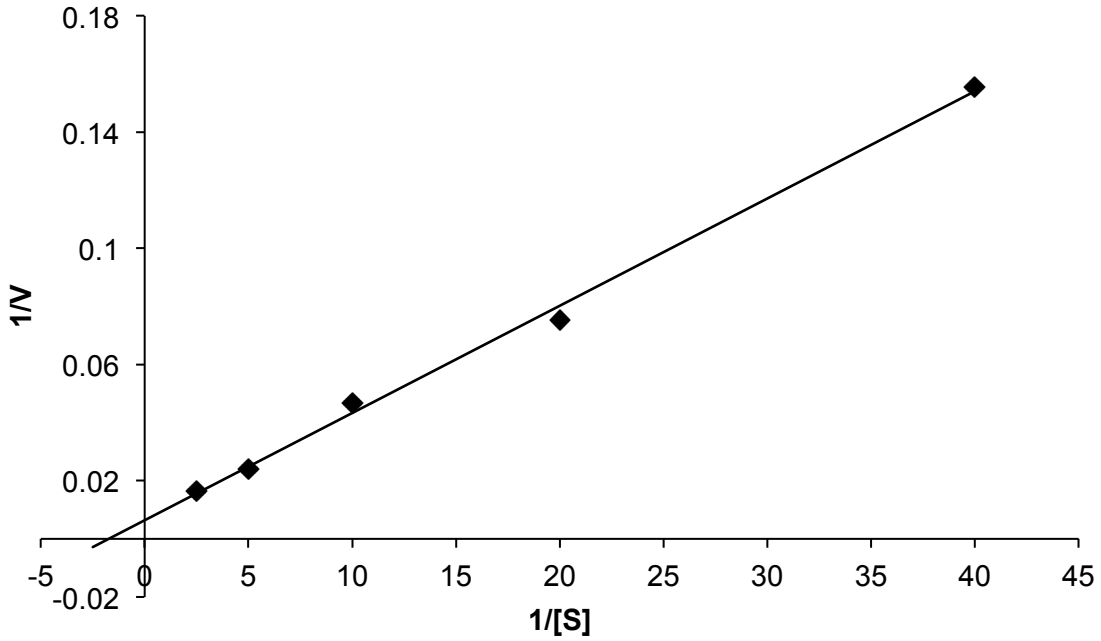


Figure 3.15. Double reciprocal plot for *E. coli* acyl-CoA dehydrogenase assayed from crude protein extract.

Acyl-CoA oxidase

Activity for this enzyme was measured using a coupled assay, the first part of which was adapted from an assay described by Lazarow and DeDuve, 1976, but instead of following reduction of its cofactor NAD at 340 nm as described in the paper, it was decided to couple this to the peroxidase assay (Polis and Shmukler, 1953), which follows the conversion of pyrogallol plus peroxide produced by acyl-CoA oxidase into purpurogallin and water, which brings about a colour change that can be followed spectrophotometrically at 430 nm (Fig. 3.16).

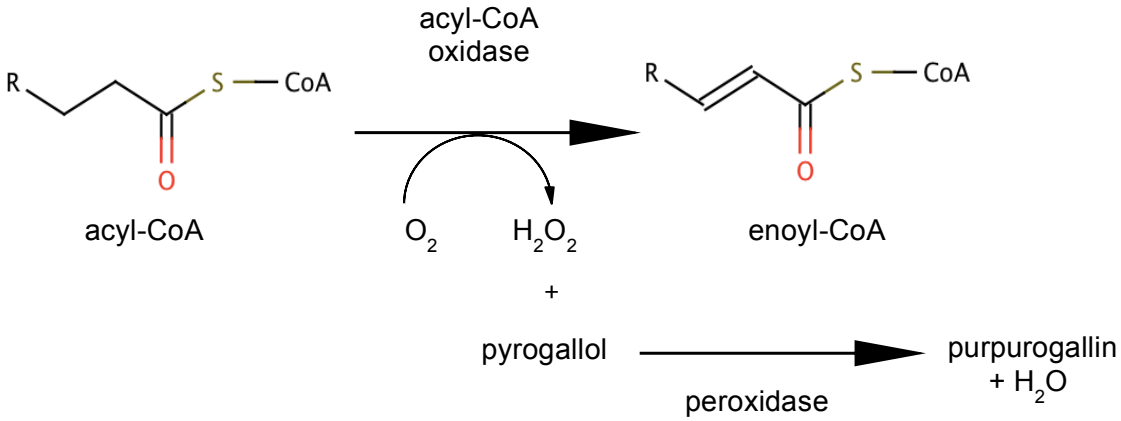


Figure 3.16. Mechanism of acyl-CoA oxidase coupled assay method.

As a positive control, crude extracts of 2 day old cress seedlings, *L. sativum* were assayed, as the β-oxidation pathway in germinating seedlings is highly active as storage lipids in seeds are metabolized during growth (Baker et al. 2006).

The average initial activity of cress acyl-CoA oxidase was found to be 26 nmol purpurogallin produced per mg of protein in crude extract per minute. In comparison, the activity in the three strains of cyanobacteria tested was negligible (Fig. 3.17).

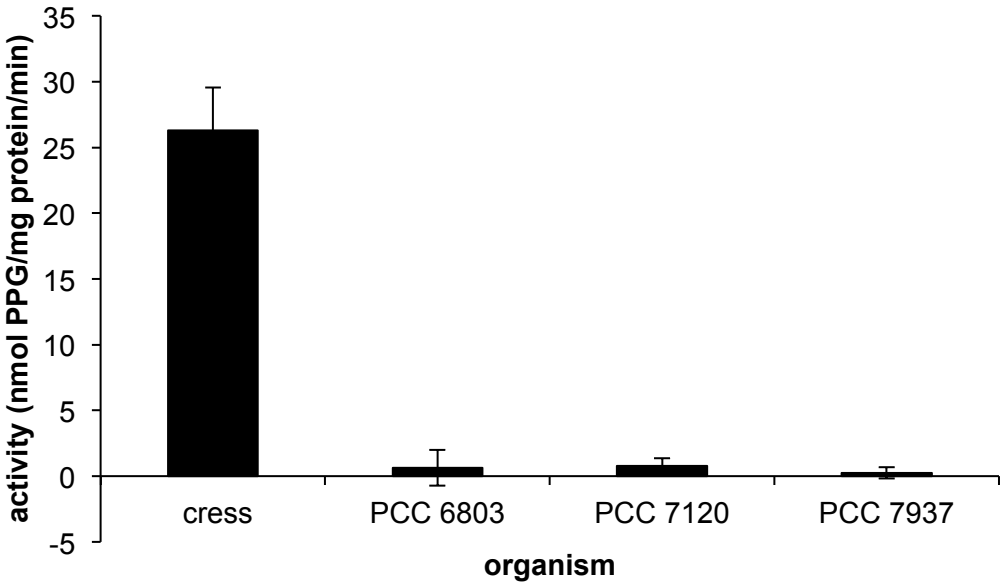


Figure 3.17. Comparison of initial rates of acyl-CoA oxidase activity as quantified by nmol of purpurogallin produced per mg of protein in crude extract per minute in cress, PCC 6803, PCC 7120 and PCC 7937. Error bars show S.E. n = 3.

3.1.5 Metabolism of ^{14}C-labelled fatty acids in cyanobacteria

In order to determine if cyanobacteria are able to break down fatty acids at all, a pulse-chase experiment was designed. This involved feeding PCC 6803, PCC 7120 and PCC 7937, as well as positive control *E. coli*, 1-^{14}C-decanoic acid and looking for resulting compounds that had picked up the label over a time period of 20 h. If breakdown products such as CO_2, sugars or other aqueous compounds are detected, then it can be concluded that cyanobacteria are able to break down fatty acids.

1. TLC Method development

In order to identify which compounds are found to have label incorporated, extracts of aqueous and DCM phase were separated out into their constituent components using TLC. In order to determine where on the TLC plate different classes of compound migrate to using various solvent systems, it was necessary to either run standards or fractionate radiolabelled samples using specific protocols that separate out different compounds into their known classes.

In order to determine which compounds were separated on solvent system 1 (2.9.6) - petroleum ether/ DCM/ acetic acid, a DCM extract from PCC 6803 that has been fed 1,2-^{14}C-acetate was fractionated using Ag^+ ion column chromatography. Using a standardized protocol, it is known that fraction 3, eluted with DCM, contains alkanes, esters and aldehydes; fraction 4, eluted with 10% acetone in DCM, contains carboxylic acids (including fatty acids), alkenes, diols and epoxides; the final fraction, eluted with acetone, contains phytols and complex lipids. Therefore it was possible to determine where on the TLC plate that these compounds migrated to (Fig. 3.18). The unfractionated sample produced three spots, one that remained at the origin, a faint band that migrated approximately 0.5 cm from the origin (indicated with an arrow) and a spot that migrated with the solvent front. Fractionation of these spots revealed that the spot that migrates with the solvent front to be either an alkane, ester, aldehyde or mixture thereof. As PCC 6803 is known to produce alkanes, it seems likely that this

was composed primarily of these compounds. The faint band that migrates to approx. 0.5 cm from the origin was determined to be a carboxylic acid, alkene, diol or epoxide or mixture thereof. As acetate can easily feed into the fatty acid biosynthetic pathway, it seems likely that this band is composed of fatty acids primarily, a very faint band can also be seen at the solvent front, this is most likely alkene. The final fraction that remains at the origin is likely composed of both phytols and complex lipids, as these are found both in great abundance in cyanobacteria, this spot also shows up coloured on the TLC plate, so accessory pigments are probably also found in this spot. These data also give evidence that acetate is a precursor to heptadecane in PCC 6803.

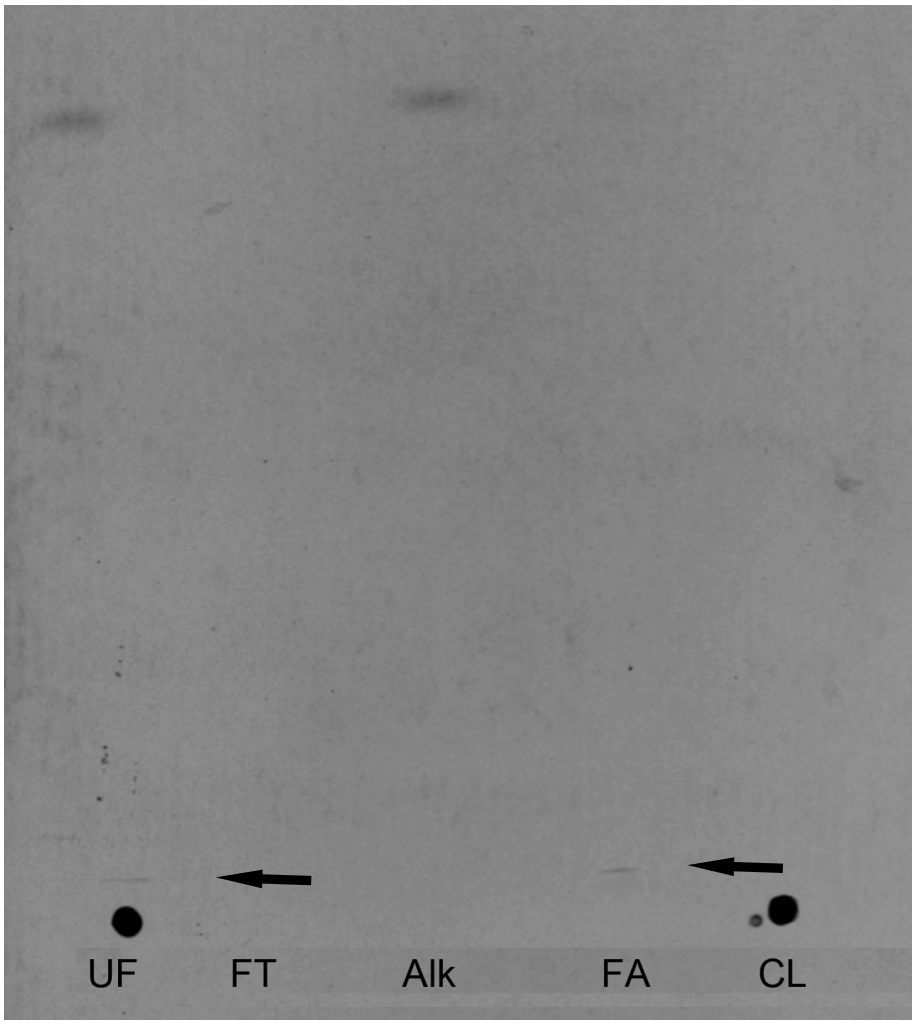


Figure 3.18. Autoradiogram of TLC plate, showing 1,2-^{14}C-acetate fed DCM extract of PCC 6803 that had been fractionated on a Ag^{+} ion column. UF = unfractionated sample; FT = initial flowthough/ wash containing no compounds; Alk = fraction containing alkanes; FA = fraction containing fatty acids; CL = fraction containing complex lipids, pigments and phytols. Arrows indicate faint fatty acid bands.

In order to determine which compounds were separated on solvent system 2 (2.9.6) - acetone/ toluene/ water, the final fraction from Ag^+ ion column chromatography was loaded onto a silica column and fractionated as described in methods. In the first fraction, eluted using DCM, neutral lipids such as hydrocarbons, triacylglycerides and fatty acids, no activity could be found (Figure 3.19), as fatty acids and hydrocarbons had been removed by the Ag^+ column previously. However, a very high proportion of activity was found in fraction 2, eluted using a DCM/ MeOH mixture that removes glycolipids (including sulfolipids). A small amount of activity could be detected in the final fraction that was eluted from the column with methanol, and contains phospholipids.

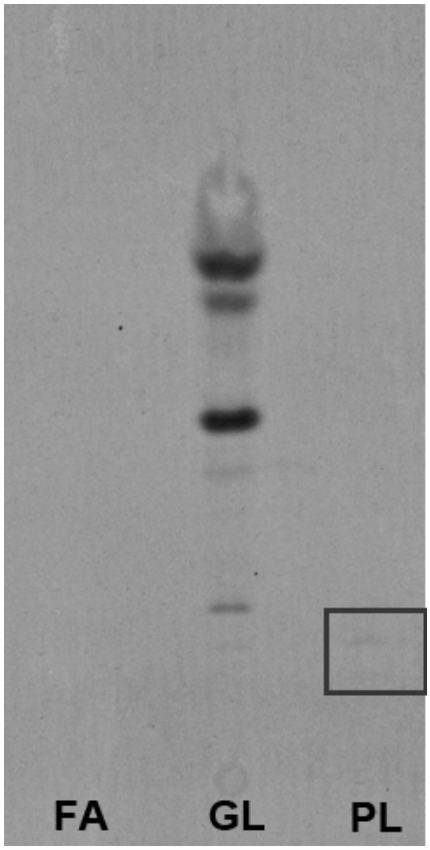


Figure 3.19. Enhanced autoradiogram of complex lipid spot from Fig. 3.26 that had been fractionated using Si column chromatography. FA = fraction containing fatty acids; GL = fraction containing glycolipids; PL = fraction containing phospholipids. Phospolipid spot indicated by square.

In order to obtain a more defininitive confirmation of where fatty acids elute using this solvent system, strain PCC 7937 was fed 1-^{14}C-decanoic acid, DCM phase extracted and subjected to the same Si column chromatography as described above, this time with no Ag^+ ion prefractionation. Additionally, a small aliquot of 1-^{14}C-decanoic acid was also run on the TLC plate to give an even clearer indication of where fatty acids migrate to using this solvent system and give some confirmation that the fractionation technique works. From Fig. 3.20, it is clear that fatty acids migrate with the solvent front. Glycolipid and phospholipid migration patterns were also similar, even in a different cyanobacterial species, giving further confirmation of the reliability of this solvent system.

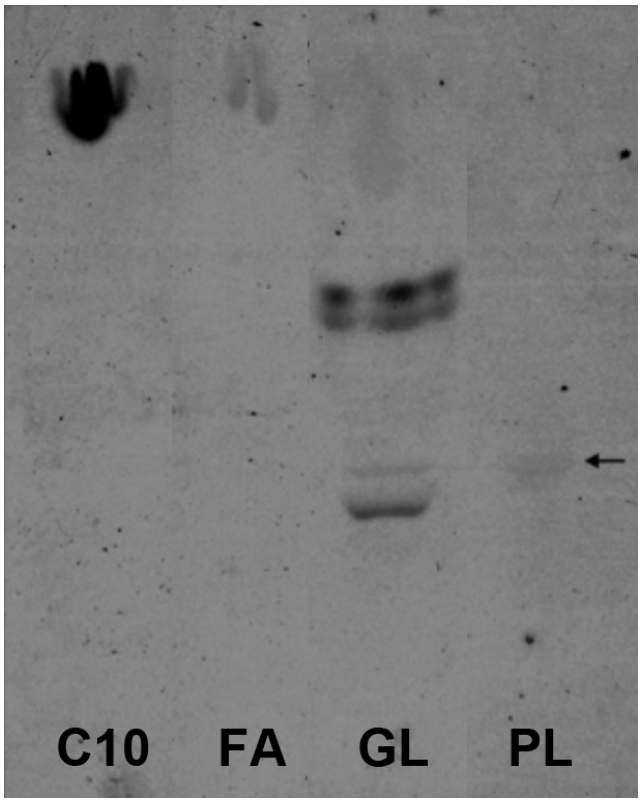


Figure 3.20. Autoradiogram of 1-^{14}C-decanoic acid fed DCM extract of PCC 7937 that had been fractionated on a Si column and ran on acetone/ toluene/ water solvent system. C10 = of 1-^{14}C-decanoic acid alone; FA = fraction containing fatty acids; GL = fraction containing glycolipids; PL = fraction containing phospholipids. Phospholipid spot indicated by arrow.

Solvent system 3 utilised an ethanol/water solvent system to separate compounds in the aqueous phase. Initially it was decided to use an ethanol/ 16.1 M ammonia solution as this ran faster, however it was found that the ammonium component of this solvent system neutralized the acidic components of the aqueous phase (e.g. organic acids and fatty acids), so that they could not be detected using pH indicator-type stains. A range of standards were ran on this solvent system listed in table 3.8. Six different staining methods were tested:

1. Silver nitrate (stains compounds containing C=C and C=O)

2. Iodine (stains compounds containing C=C and C=O)

3. Primuline (compounds containing C=O fluoresce under UV light)

4. Aniline-diphenylamine (stains sugars)

5. Bromophenol blue (pH indicator – will stain acids and sugars – darker blue stain observed from sugars and yellow stain observed from organic acids)

6. Ninhydrin (stains amino acids)

It was found that ninhydrin was most effective for staining amino acids (Fig.3.21, A) and that bromophenol blue was the most effective for staining organic acids and sugars (Fig 3.21, B).

Table 3.8. Amino acid, organic acid and sugar standards ran on ethanol/water solvent system, with corresponding R_f values listed.

Compound	**R_f value**
Methionine	0.610
Tyrosine	0.476
Histidine	0.410
Serine	0.275
Proline	0.171
Citric acid	0.111
Succinic acid	0.317
Fumaric acid	0.427
Malic acid	0.212
Acetic acid	0.624
Sucrose	0.264
Fructose	0.386

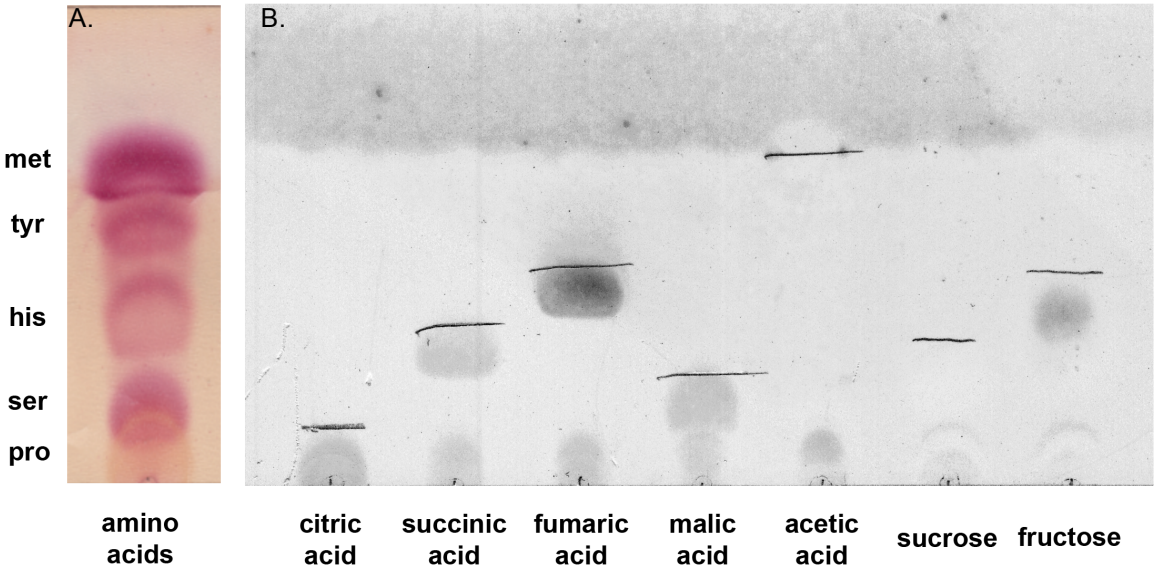


Figure 3.21. TLC plate ran on solvent system ethanol/ water 7:3. **A.** TLC of amino acid standards mix stained with ninhydrin. **B.** TLC of organic acids and sugars stained with bromphenol blue. Note negative staining for acetic acid and sucrose. Spots visible near origin were due to sample overloading.

2. LC-QToF-MS method development for detection of complex lipids.

Whole lipid extracts from PCC 7937 and *E. coli* were fractionated on a Si column for complex lipids as described in 2.9.5, but instead ran on LC-QToF-MS as described in 2.9.8. Structural elucidation of glycolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG), and the phospholipids phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) was possible by examining MS/MS data, based on studies conducted by Bauersachs et al. 2009; Ekroos et al. 2002 and Sturt et al. 2004.

An MGDG precursor ion was found to be present as a formate ($M+COO^-$) adduct at 795.5332 m/z (Fig. 3.22). Collision induced dissociation (CID) yielded two free fatty acid product ions, at 253.22 and 277.22 m/z that match to 16:1 and 18:3 acyl chains and the formate adduct at 44.998 m/z. Masses were within 7 ppm accuracy for precursor ion and 4 ppm for product ions. Positions of double bonds in acyl chains could not be determined, but are probably in the *cis*-9 for 16:1 and *cis*-9-12-15 or *cis*-6-9-12, as these are most commonly found in nature. This was the only type of MGDG that could be detected in this extract.

A DGDG precursor ion was found to be present as a formate adduct (Fig. 3.23) CID always gave the characteristic DGDG head group ion at 415.1457 m/z and two H_2O neutral losses of that ion giving 397.13 m/z and 379.1 m/z. 16:1 and 18:3 acyl chains were also detected at 253.22 m/z and 277.22 m/z. All masses were within 7 ppm mass accuracy. Positions of double bonds in acyl chains have not been determined but are likely in the *cis*-9 for 16:1 and *cis*-9-12-15 or *cis*-6-9-12, as these are most commonly found in nature.

A SQDG precursor ion was present as (M-H) at 791.498 m/z (Fig. 3.24). CID always yielded a sulfoquionovosyl head group with a neutral loss of water to give 225.007 m/z. Mass accuracy was within 7 ppm for precursor ion and 2 ppm for product ion. Note that the composition of acyl chains cannot be determined using these MS/MS data, for

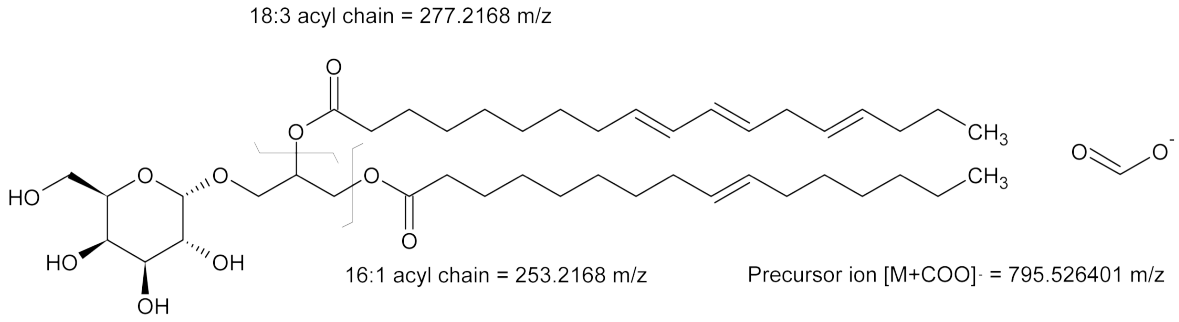
example SQDG(32:1) could have 16:1/16:0 acyl chains, but it could also have 30:1/2:0 acyl chains, although it logically seems more likely that the former would be correct, as these lengths of acyl chain are more common in other classes of complex lipid, however it cannot be shown experimentally that this is the case.

A PC precursor ion was present in positive polarity at 758.5497 m/z (M+H) (Fig. 3.25). CID always yielded a phosphocholine headgroup at 184.073 m/z. Mass accuracy was within at least 26 ppm for precursor ion and 12 ppm for product ion. As with SQDG, composition of acyl chains was not possible using the MS/MS data generated.

A PG precursor ion was found to be present as (M-H) at 747.5161 m/z (Fig. 3.26). CID yielded a characteristc head group ion minus the glycerol moiety at 153.996 m/z as well as two acyl chain product ions at 281.248 m/z and 255.2327 m/z corresponding to 18:1 and 16:0. Position of double bond in 18:1 could not be determined, but is likely to be in the *cis*-9 position, as this is most commonly found in nature. Mass accuracy was within at least 11 ppm for precursor ion and 5 ppm for product ions.

A PE precursor ion was found to be present as (M-H) at 452.2711 m/z (Fig. 3.27). CID yields a PE head group with m/z of 196.037 and acyl chain at 255.2337 m/z corresponding to 16:0. Mass accuracy was within at least 16 ppm for precursor ion and 6 ppm for product ions.

A.



B.

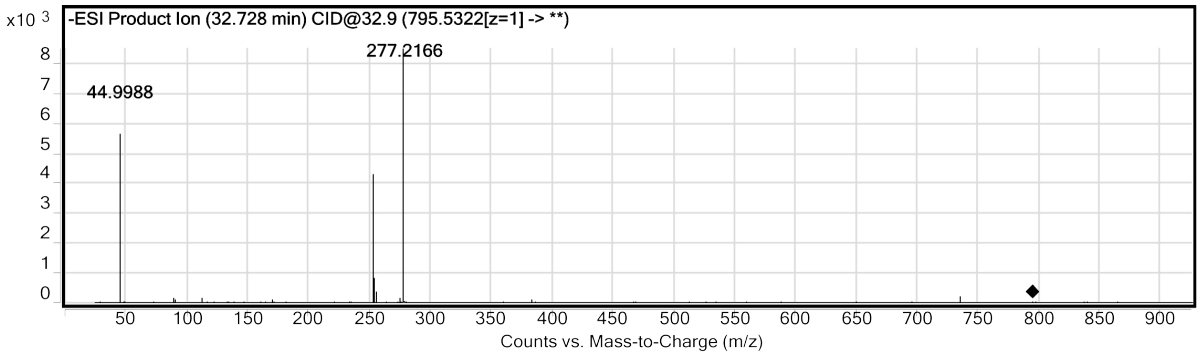


Figure 3.22. Probable identification of MGDG. **A** – Structure of MGDG(16:1/18:3) plus formate adduct, showing where ion fragments after CID and their resulting acyl chain product masses. **B** – MS/MS spectrum, showing precursor ion (diamond), acyl chain peaks at 277.2152 m/z and 253.2179 m/z and formate adduct at 44.9988 m/z.

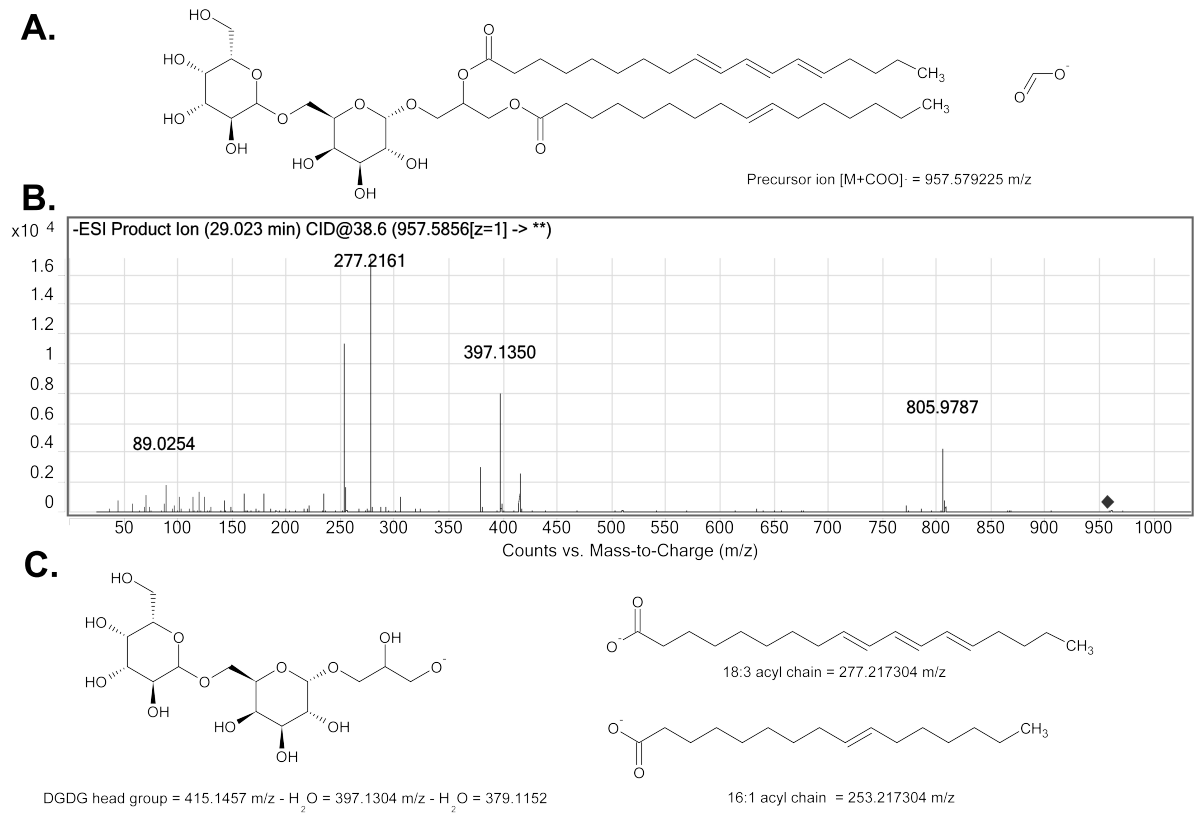
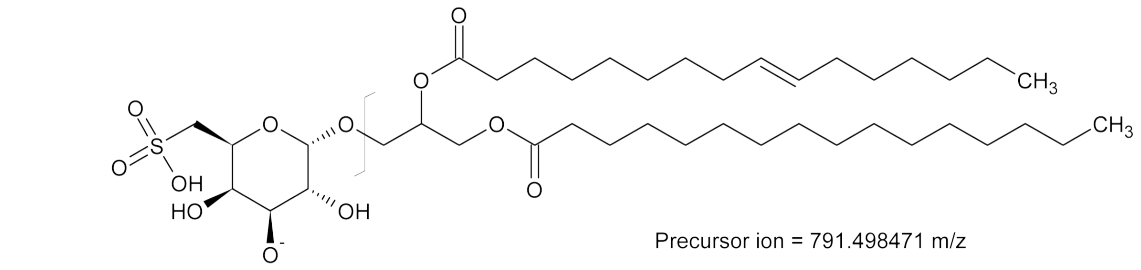


Figure 3.23. Probable identification of DGDG. **A** – Structure of precursor ion as formate adduct. **B** – MS/MS spectrum of DGDG, showing precursor ion as diamond; 3 head group product ions at 415.1457 m/z, 397.13 m/z and 379.1 m/z; and 2 acyl chain product ions at 277.2152 m/z and 253.2179 m/z. **C** – Structure and exact masses of product ions resulting after CID.

A.



B.

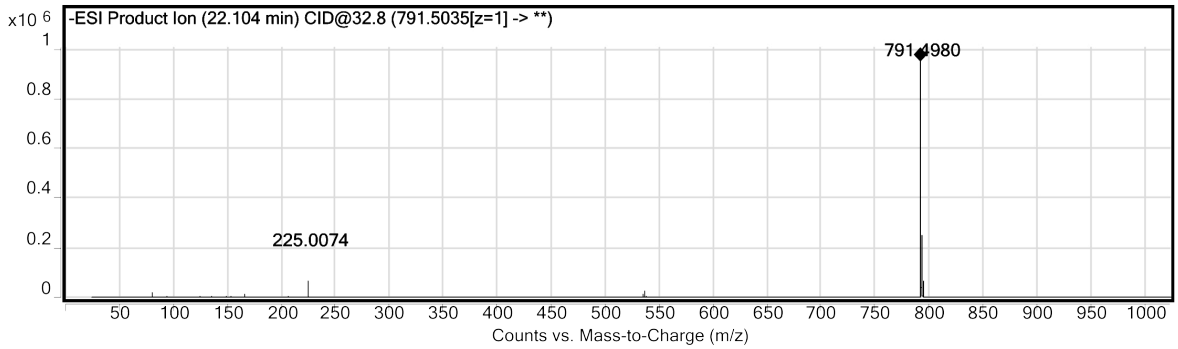
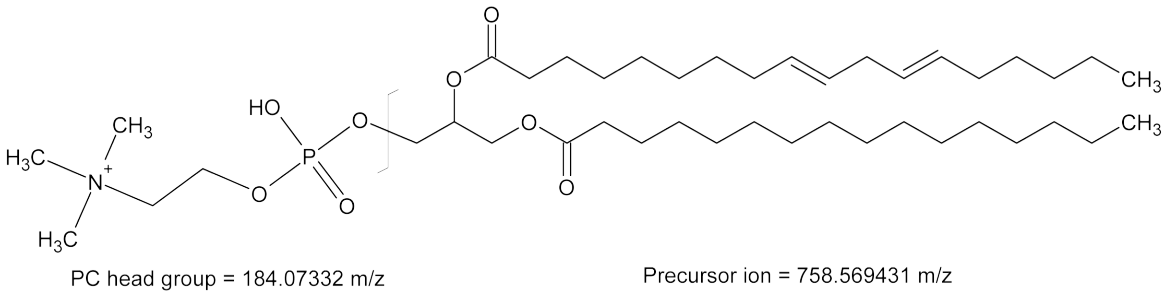


Figure 3.24. Probable identification of SQDG. **A** – Structure of precursor. Line shows where ion fragments to give resulting product m/z plus neutral loss of water at 225.0069 m/z. **B** – MS/MS spectrum, showing precursor ion indicted by diamond and product ion at 225.0074 m/z.

A.



B.

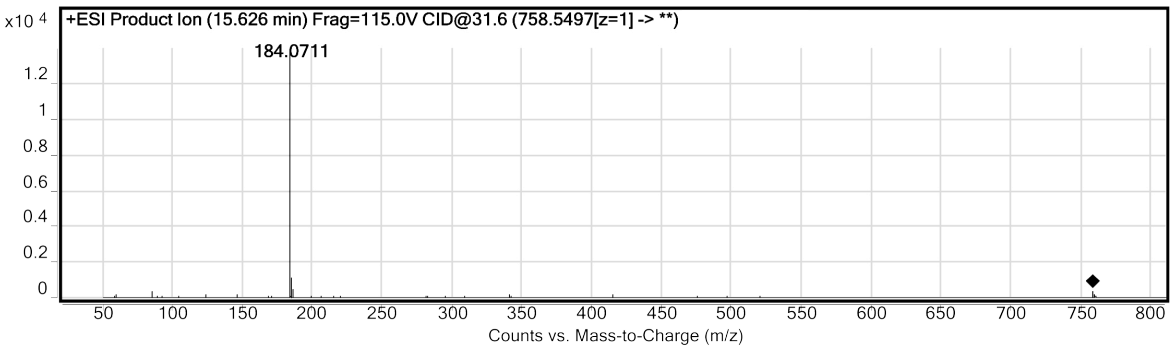


Figure 3.25. Probable identification of PC. **A** – Structure of PC precursor in positive polarity at 758.5694 m/z and its respective head group product ion at 184.0733 m/z. **B** – MS/MS spectrum, showing precursor ion indicted by diamond and product ion at 184.0711 m/z.

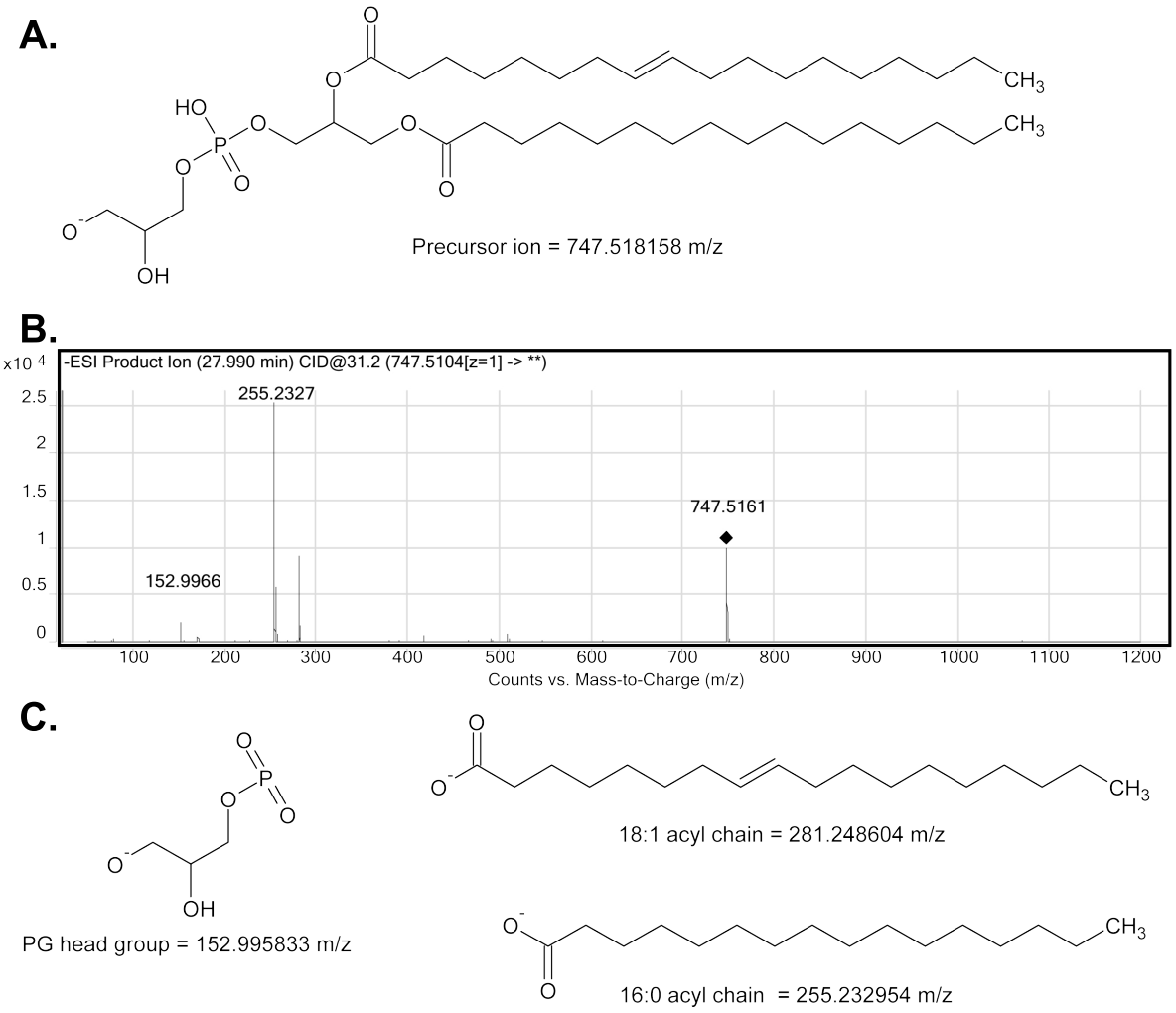


Figure 3.26. Probable identification of PG. **A** – Structure of precursor ion. **B** – MS/MS spectrum of PG, showing precursor ion at diamond; head group product ion at 152.9966 m/z and 2 acyl chain product ions at 281.2452 m/z and 255.2327 m/z. **C** – Structure and exact masses of product ions resulting after CID.

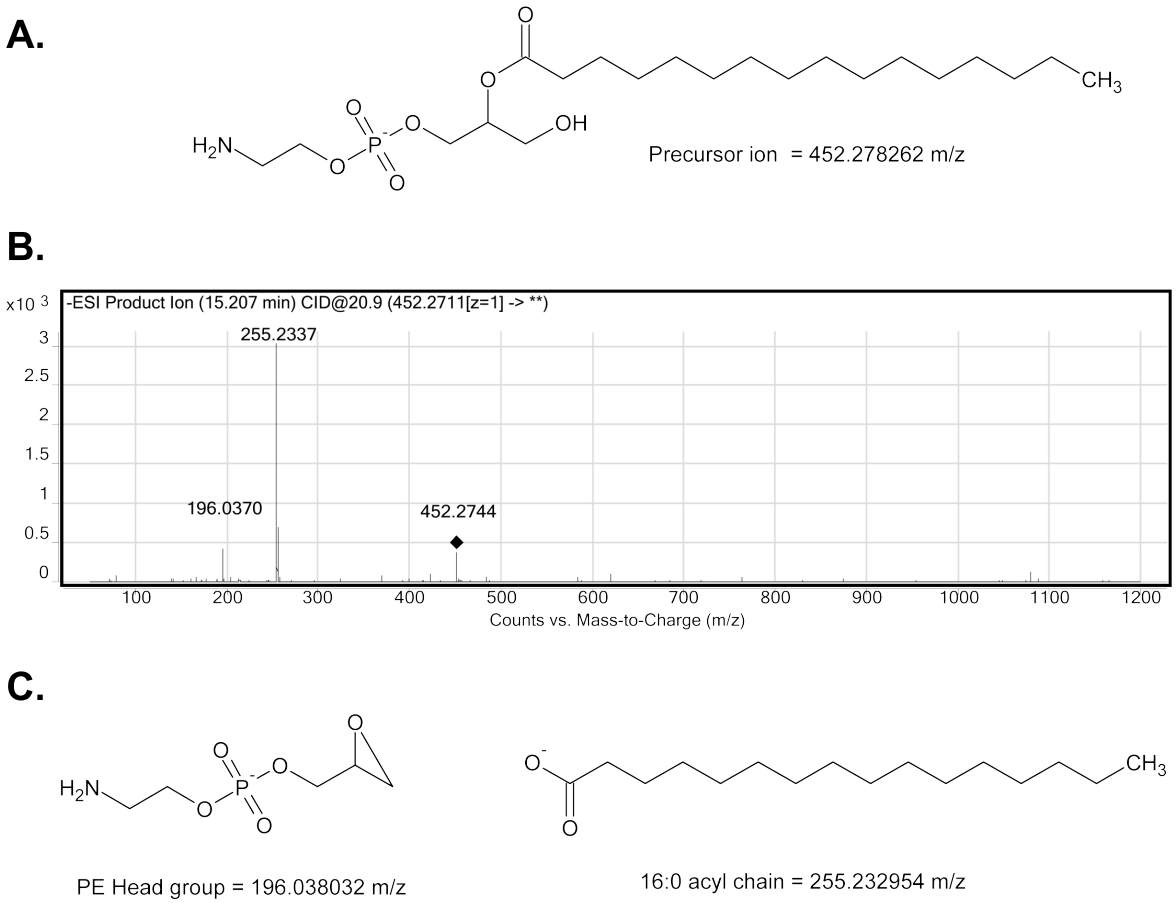


Figure 3.27. Probable identification of PE. **A** – Structure of precursor ion. **B** – MS/MS spectrum of PG, showing precursor ion at diamond; head group product ion at 196.037 m/z and acyl chain product ion at 255.2337 m/z. **C** – Structure and exact masses of product ions resulting after CID.

3. Pulse chase labeling experiment

Cyanobacterial strains PCC 6803, PCC 7120 and PCC 7937, as well as *E. coli* as a positive control were fed 1-^{14}C-decanoic acid for 1 h in the dark, before being resuspended in label-free medium. Samples were taken at 1 h, 4 h, 8 h and 20 h. In additon, non-radiolabelled decanoic acid was fed to cyanobacterial and *E. coli* cultures in parallel in order to assist in compound identification by LC-QToF-MS. Cyanobacteria were fed in the dark in order to prevent CO_2 being recycled into other compounds by photosynthesis.

Samples were extracted as described in methods section 2.9 and subject to analysis by scintillation counting, TLC and LC-QToF-MS in order to detect resulting products arising from fatty acid biosynthesis or metabolism.

a. Uptake of label from medium

E. coli was observed to take up on average (n=3) 71% of 1-^{14}C-decanoic acid from the medium after 1 hour (Fig. 3.28, F). After 8 h radiolabel was seen to increase in media from a low of 11.0% to 16.1% after 20 h, indicating that label was being re-introduced back into the medium from *E.coli.* No radioactivity was detected in aqueous compounds on the TLC autoradiogram (Fig. 3.29, A), indicating that the label was likely incorporated into a gas dissolved in the medium.

PCC 6803, PCC 7120 and PCC 7937 in the dark were much better able to take up 1-^{14}C-decanoic acid from their medium than *E.coli*, with 10.6% of total radiolabel remaining after 1 hour in PCC 6803. Negligible label was observed in the media thereafter (<0.6 % of total label). PCC 7120 and PCC 7937 were not as efficient as PCC 6803 at taking up 1-^{14}C-decanoic acid from their medium, with 15.6 % and 13.9 % of total label remaining after 1h respectively, but after 20 h dropping to similar levels observed in PCC 6803 (1.4 % and 0.4 % total label respectively), indicating no label was being released back into the medium in cyanobacterial strains unlike in *E. coli.*

b. Label released as CO_2

$^{14}CO_2$ was released from 1-^{14}C-decanoic acid by *E. coli* initially at a rate of 2.8 % of total label per h after 1h (Fig. 3.28, B), increasing to a maximum of 4.5 % total label /h after 4h before decreasing to 1.4 % total label /h, total label incorporated reaching 47 % CO_2 after 20 h (Fig. 3.28, A).

$^{14}CO_2$ was released from PCC 6803, PCC 7120 and PCC 7937 at a much lower rate, between 0.3 % and 0.7 % total label /h for PCC 6803 and <0.1 % to 0.7 % total label /h for PCC 7120 and 0.1 % to 0.3 % total label /h for PCC 7937. Total $^{14}CO_2$ released from 1-^{14}C-decanoic acid in cyanobacteria did not exceed 1.3 %, 17- fold lower than observed in *E. coli.*

c. Label incorporated into aqueous phase

In *E. coli* 53% of total radioactivity from 1-^{14}C-decanoic acid was incorporated into compounds in the aqueous phase, decreasing to 34.7% after 4h and staying at a similar level for the remainder of the timecourse (Fig. 3.28, C). Analysis of this fraction using TLC revealed label was incorporated into only one spot that moves with the solvent front (Fig. 3.29, B) at 1 h, but was observed to disappear, again suggesting that the radioactivity observed in this fraction after 1 h could be CO_2 or another gaseous compound that would not run on a TLC plate.

Analysis of the aqueous extracts from *E.coli* cultures at 1 h that were fed unlabeled decanoic acid and ran in parallel on the same TLC plate using LC-QToF-MS revealed that the major peaks present in the total ion chromatogram (TIC) to have the same negative ion masses (M-H) as decanoic acid, palmitic acid and stearic acid (171.2523, 256.2301 and 284.2713 m/z) (Fig. 3.33).Other peaks for different fatty acids were also present at lower abundance (Fig. 3.34 and Table 3.10). No amino acids, organic acids or sugars were observed to have incorporated label.

The average (n=3) proportion of label from 1-^{14}C-decanoic incorporated into aqueous compounds after 1 h was 40.4% of total for PCC 6803, 34.7 % for PCC 7120 and 35.1 % for PCC 7937, these values remained close to these levels (within standard error) for the remainder of the timecourse.

Analysis of these fractions using TLC revealed label incorporated remained present throughout the timecourse (Figs. 3.29, B; 3.30, B and 3.31, B.), unlike in *E. coli*. Analysis of corresponding spots in non-radioactive decanoic acid fed samples by LC-QToF-MS revealed these compounds had the same (M-H) as decanoic acid, palmitic acid and stearic acid (Fig. 3.33) (171.2523, 256.2401 and 284.2713 m/z), the abundance of decanoic acid was much higher in cyanobacteria than in *E. coli* (Table 3.9), higher peaks observed for long chain fatty acids in PCC 7937 could have been due to the presence of a long chain thioesterase (EC: 3.2.1.14) that this organism has computational annotation for, but is lacking in the other two strains of cyanobacteria; this thioesterase acts on acyl-ACPs with chain lengths from 12 to 18 carbons (Ohlrogge et al. 1978). Peaks for other fatty acids were also present at lower abundance (Fig. 3.34 and Table 3.10). Odd chain length fatty acids could be detected in TLC scrapings from both *E. coli* and cyanobacteria, it is likely that these are methyl-branched or cyclo- fatty acids, however this could not be confirmed by LC-QToF-MS.

In cyanobacterial scrapings, 2 peaks were observed in the chromatogram for 17:0, indicating that one of these may be a branched 16:0 fatty acid and the other either a straight chain. In *E. coli* the 17:0 peak was at a different retention time to both of these peaks, indicating it could be a cyclo-17:0 peak.

d. DCM phase

10.5% of total label from 1-^{14}C-decanoic acid was found to be incorporated into the DCM phase, which contains lipids and other hydrophobic compounds, in *E. coli* after 1 h, rising to 43.9% after 8 h, before dropping down to 27.7% after 20 h (Fig. 3.28, D). The drop in radioactivity in DCM phase coincided with a rise in total $^{14}CO_2$ detected

(Fig. 3.28, A). Label from 1-^{14}C-decanoic acid was rapidly incorporated into DCM phase compounds in the cyanobacteria, with 48.1% - 49.5% average of total activity detected in this phase after 1 h (Fig. 3.28, D). This continued to rise to 57.9% - 64.2% of total label after 4 h before remaining at a similar level (within standard error) for the remainder of the timecourse. TLC autoradiograms also show that fatty acid disappeared in *E. coli* after 1h (Fig. 3.29, D), but remains in the cyanobacterial samples (Figs. 3.30, D; 3.31, D and 3.32, D).

e. Analysis of complex lipids in *E. coli*

Analysis of DCM fractions using TLC with acetone/toluene/water 90:30:1 v/v/v solvent system (Figs. 3.29, C; 3.30, C; 3.31, C and 3.32, C) showed that in cyanobacteria many more compounds were detected than in *E.coli*. Spots that migrated with the solvent front in this solvent system have been shown to be fatty acids (Fig. 3.20) and spots that migrate roughly ½ the way across the plate were complex lipids. However it was difficult to resolve phospholipids from glycolipids using TLC alone, so LC-QToF-MS was used to identify complex lipids where activity was incorporated from decanoic acid that had been fed to samples.

Analysis of non-radiolabeled decanoic acid fed cultures of *E. coli* using LC-QToF-MS revealed that all of the label detected in the single spot on the TLC plate using the solvent system described above was incorporated into phospholipids PC, PG and PE (Table 3.11). PG and PE have been reported in *E. coli* previously by similar methods (Ekroos et al. 2002) however, it is widely acknowledged in the literature that PC is not present in *E. coli* (Cronan and Vagelos, 1972), yet it can be detected in these TLC scrapings - it seems unlikely that these precursor and product ion masses could be another compound as their mass accuracy values are high (1.0 to 8.8 ppm) and the sample had already been pre-purified by TLC; carryover contamination in the instrument can also be ruled out as a blank was run between samples and the injection needle washed. PC is the most common phospholipid in eukaryotes and is synthesized

from CDP-choline and 1,2-diacylglycerol, whereas in the few bacterial species that have PC it is synthesized by methylation of PE (Ridgeway and Vance, 1988), *E. coli* is apparently lacking in the enzyme sequences for both of these pathways according to computational annotation. Further work could be carried out to determine if PC is present in *E. coli*, however this is not the primary aim of this study. Exact composition of acyl chains in PC cannot be determined, but could be 18:3/16:0 for PC(34:3), 18:2/16:0 for PC(34:2) as these acyl chains are also found in other classes of phospholipid where acyl chain composition can be determined, although Ekroos et al. 2002 found that different isobaric forms of acyl chains can exist in PE of *E. coli*, for example PE(33:1) was found to be present as PC(16:0/17:1) and PC(15:0/18:1).

All PE phospholipids in the *E. coil* samples were found to be present as lyso-PCs, this could have arisen during TLC or by in-source fragmentation, as previous profiling reports show that both chains are present in this phospholipid in *E. coli*. Also found in the PE phospholipid class was a cyclopropane 17:0 acyl chain, which are known to be present in *E. coli* and some other gram negative bacteria (Cronan et al. 1974).

f. Analysis of complex lipids in cyanobacteria

Analysis of the lower spots in non-radiolabeled decanoic acid fed cultures of PCC 6803 also show PCs and lyso-PGs as in *E. coli* (Table 3.12). In addition to phospholipids, glycolipid SQDG(16:0/0:0) was also detected. Analysis of the upper spot on the TLC plate revealed the only complex lipid detectable was DGDG(16:0/0:0). All complex lipids detected except for PCs were present as lyso lipids, having only one acyl chain present, PCC 6803 is known have both intact lipid and lyso- lipids present (Kim et al. 1999), it appears that only lyso- versions of the lipids have picked up label.

Phospholipids PC and PG could be detected in the lower spots of PCC 7120 (Table 3.13). MGDG(18:3/16:0) was present in the upper spot, no DGDG could be detected. MGDG(18:3/16:1) was detected in the upper spot of PCC 7120 (Table 3.14). 3 types of DGDG and 12 types of SQDG were found in the lower spot, but the only phospholipid

detectable was PG(16:0/16:0), it is possible that the large number of DGDGs and SQDGs were competing for ionization in source with phospolipids and so suppressed their signal.

Absolute quantification of complex lipids using LC-QToF-MS was not possible as different types of complex lipid have different ionization efficiencies and so the use of internal standards, such as ^{13}C-labelled internal standards, would be required – such compounds are not readily available and would have to be chemically or biologically synthesized and extracted, which is a very time consuming process. The complex lipid composition for each timepoint in the cyanobacteria showed no major changes over the 20 h timecourse.

g. Pellet

Negligible radioactivity arising from 1-^{14}C-decanoic acid could be detected in the pellet of all strains, as both *E. coli* and cyanobacteria show maximum 2% average of total label incorporated (Fig. 3.28, E), which was not significantly above background level.

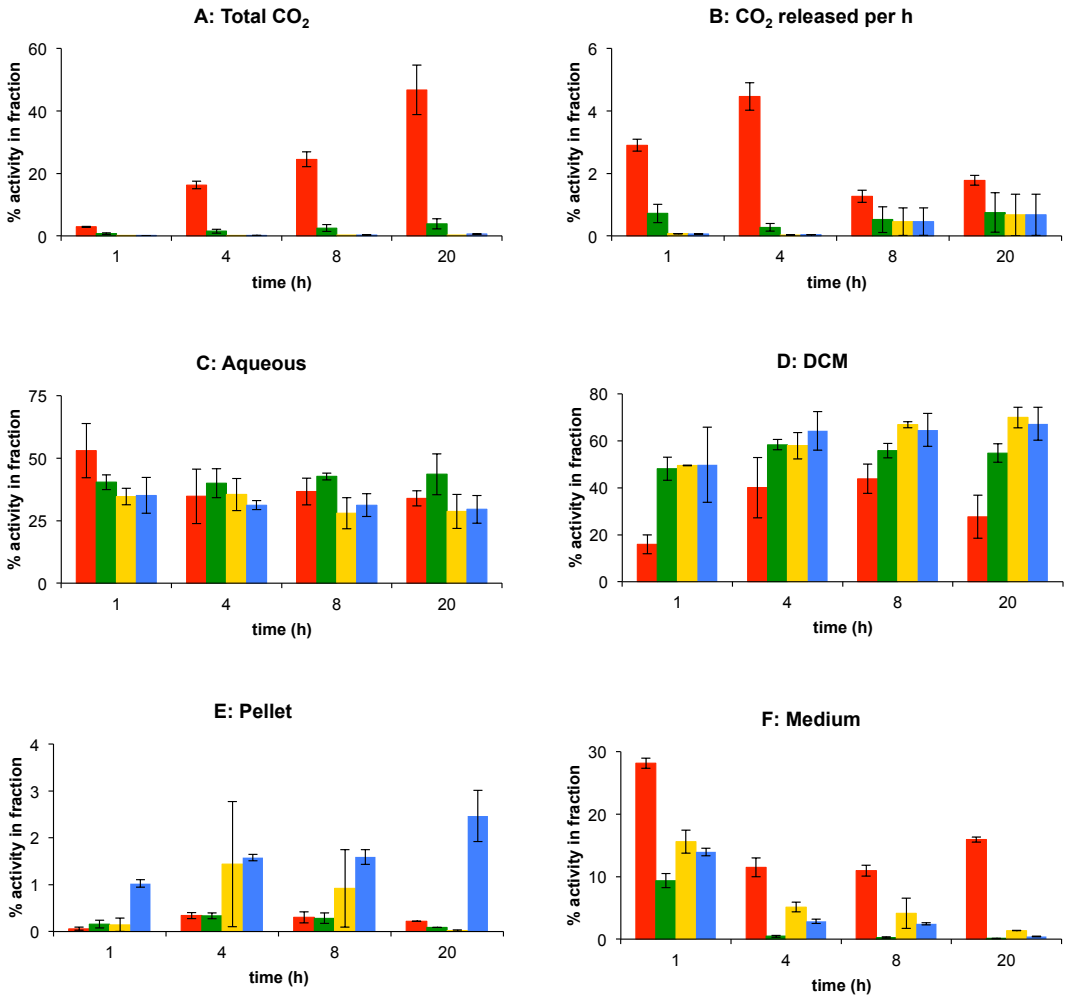
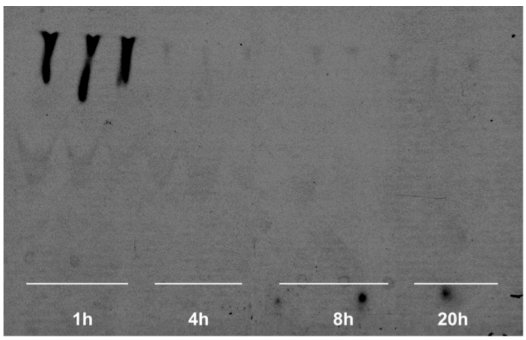
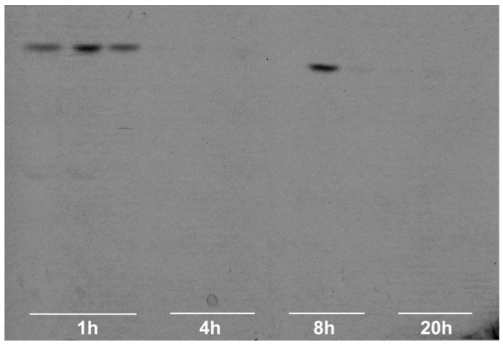


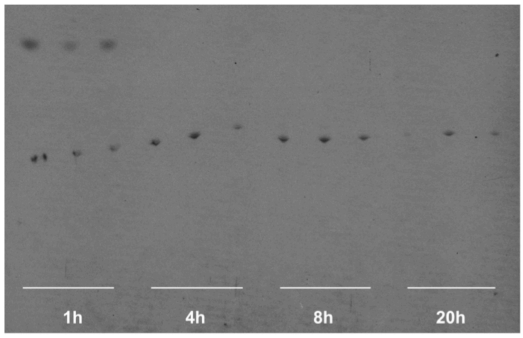
Figure 3.28. ^{14}C label from 1-^{14}C-decanoic acid fed to cultures of *E. coli* (red bars), PCC 6803 (green bars), PCC 7120 (yellow bars) and PCC 7937 (blue bars), that was released as total CO_2 (**A.**), CO_2 released per hour (**B.**); or incorporated into the aqueous phase (**C.**), DCM phase (**D.**), pellet (**E.**); or remaining in the medium (**F.**). Error bars show ± S.E. (n = 3).



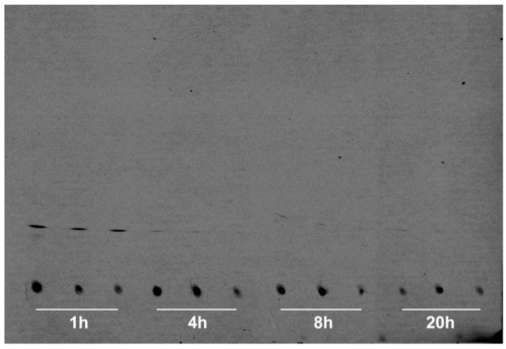
A: *E.coli* medium



B: *E.coli* aqueous

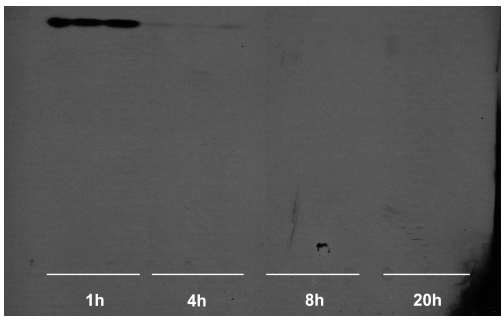


C: *E. coli* complex lipids

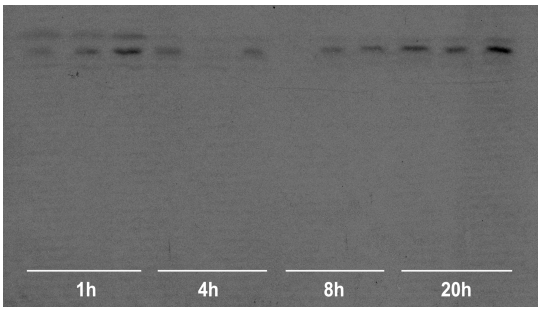


D: *E. coli* hydrocarbons

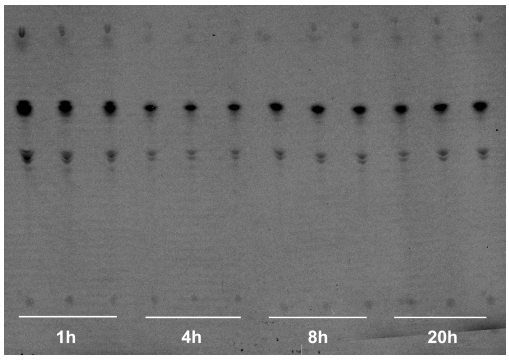
Figure 3.29**.** Autoradiograms of TLCs of medium, aqueous and DCM fractions in *E. coli.* Media and aqueous fractions separated (A and B) with ethanol/18.1 M ammonia 7:3 v/v solvent system; “complex lipids” (C) separated with acetone/toluene/water 90:30:8 v/v/v solvent system; “hydrocarbons” (D) separated with petroleum ether/DCM/acetic acid 90:10:1 v/v/v.



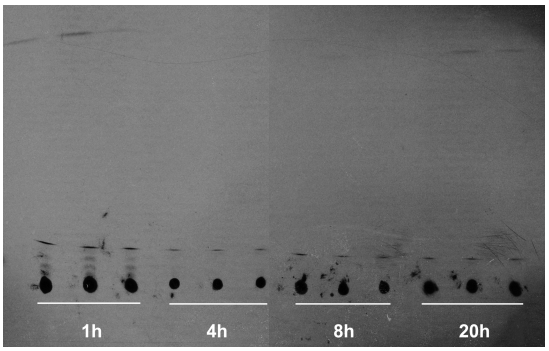
A: PCC 6803 medium



B: PCC 6803 aqueous

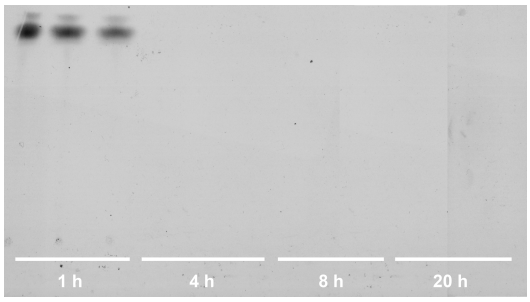


C: PCC 6803 complex lipids

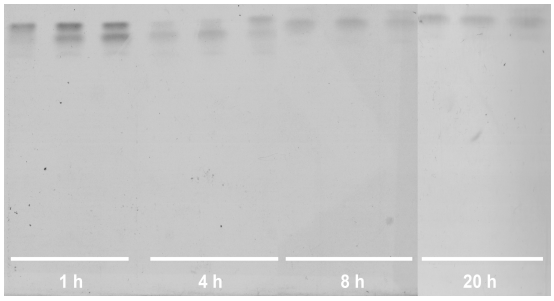


D: PCC 6803 hydrocarbons

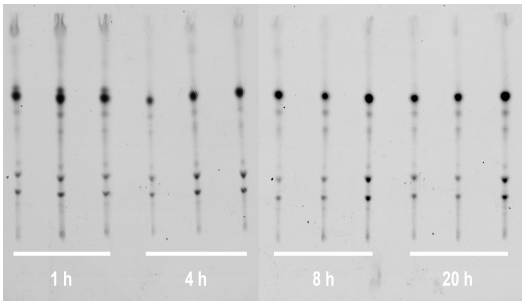
Figure 3.30**.** Autoradiograms of TLCs of medium, aqueous and DCM fractions in PCC 6803*.* Media and aqueous fractions separated (A and B) with ethanol/18.1 M ammonia 7:3 v/v solvent system; “complex lipids” (C) separated with acetone/toluene/water 90:30:8 v/v/v solvent system; “hydrocarbons” (D) separated with petroleum ether/DCM/acetic acid 90:10:1 v/v/v.



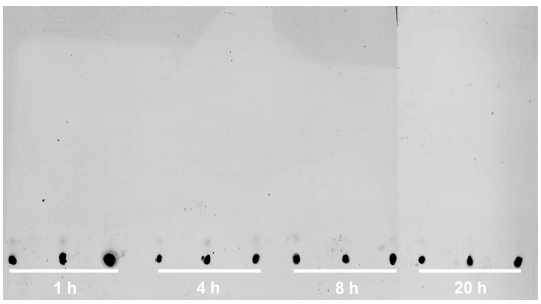
A: PCC 7120 medium



B: PCC 7120 aqueous

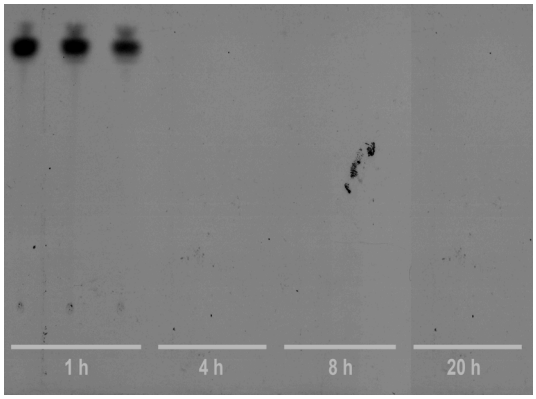


C: PCC 7120 complex lipids

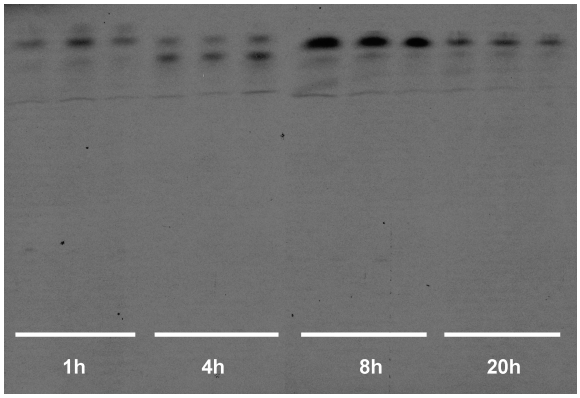


D: PCC 7120 hydrocarbons

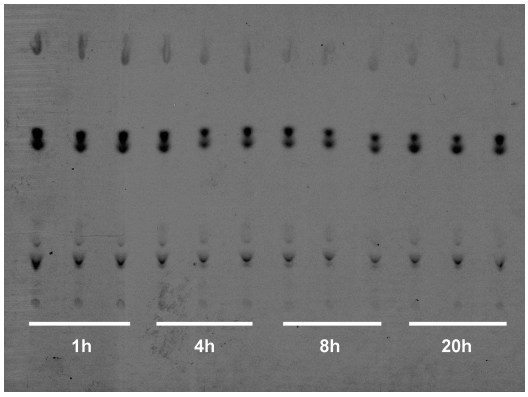
Figure 3.31. Autoradiograms of TLCs of medium, aqueous and DCM fractions in PCC 7120. Media and aqueous fractions separated (A and B) with ethanol/18.1 M ammonia 7:3 v/v solvent system; “complex lipids” (C) separated with acetone/toluene/water 90:30:8 v/v/v solvent system; “hydrocarbons” (D) separated with petroleum ether/DCM/acetic acid 90:10:1 v/v/v.



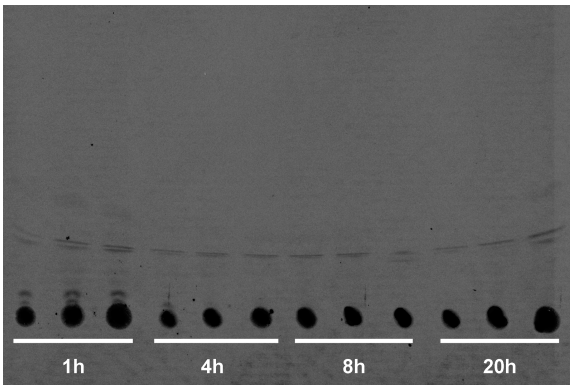
A: PCC 7937 Medium



B: PCC 7937 aqueous



C: PCC 7937 complex lipids



D: PCC 7937 hydrocarbons

Figure 3.32**.** Autoradiograms of TLCs of medium, aqueous and DCM fractions in PCC 7937. Media and aqueous fractions separated (A and B) with ethanol/18.1 M ammonia 7:3 v/v solvent system; “complex lipids” (C) separated with acetone/toluene/water 90:30:8 v/v/v solvent system; “hydrocarbons” (D) separated with petroleum ether/DCM/acetic acid 90:10:1 v/v/v.

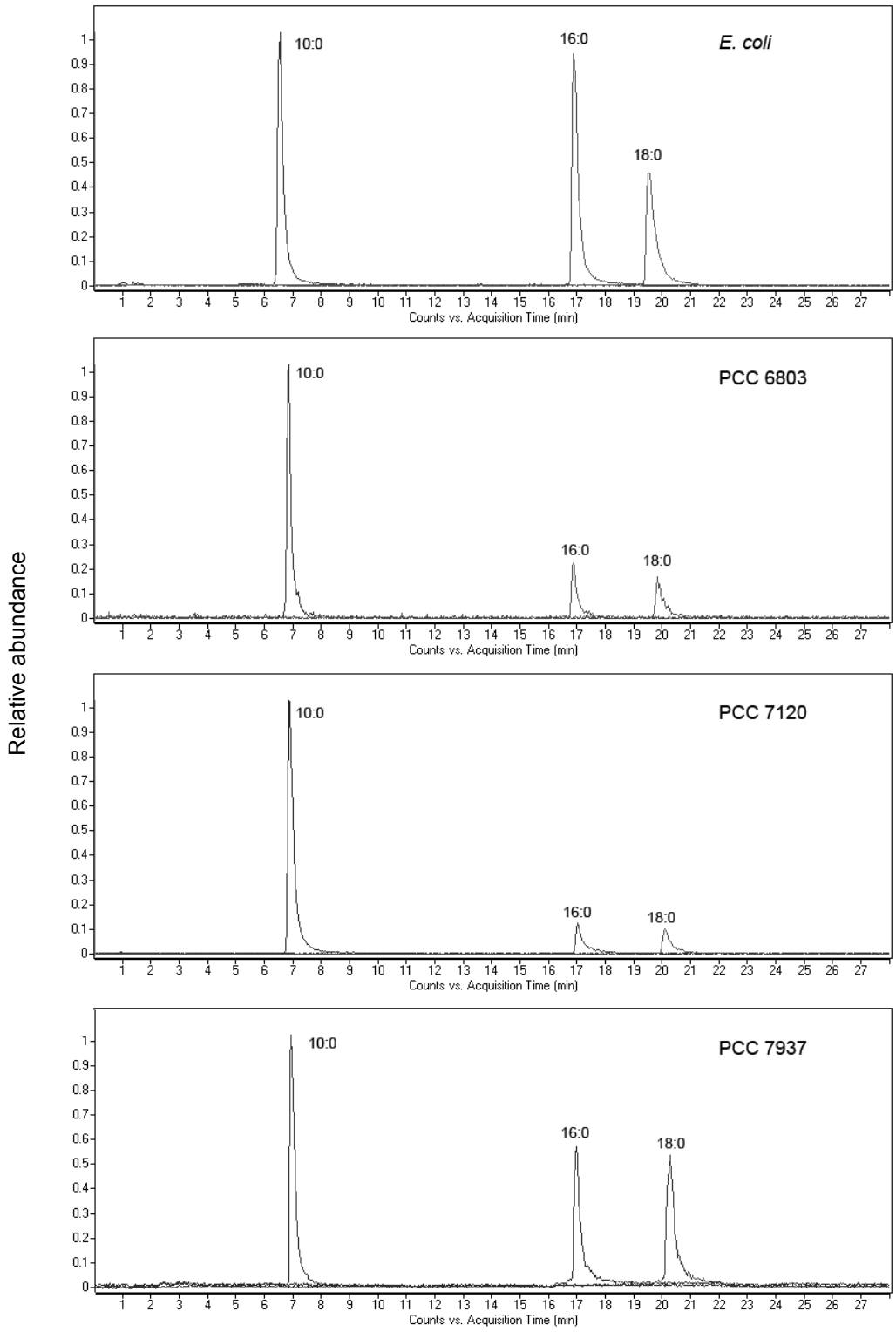


Figure 3.33. Extracted ion chromatograms for decanoic acid (10:0; 171.2523 m/z) palmitic acid (16:0; 256.2401 m/z) and stearic acid (18:0; 284.2713 m/z) taken from single QToF runs of spots where radioactivity was found to be on TLC plates of the aqueous phase in *E. coli*, PCC 6803, PCC 7120 and PCC 7937.

Table 3.9. Integrated areas of decanoic acid, palmitic acid and stearic acid peaks in *E. coli*, PCC 6803, PCC 7120 and PCC 7937 from figure 3.41.

Compound	**Integrated peak areas**			
	E. coli	**PCC 6803**	**PCC 7120**	**PCC 7937**
10:0	1,112,009	11,124,844	13,730,825	13,080,711
16:0	1,000,596	6,387,076	1,931,817	9,107,617
18:0	1,485,138	5,983,093	1,411,008	9,018,339

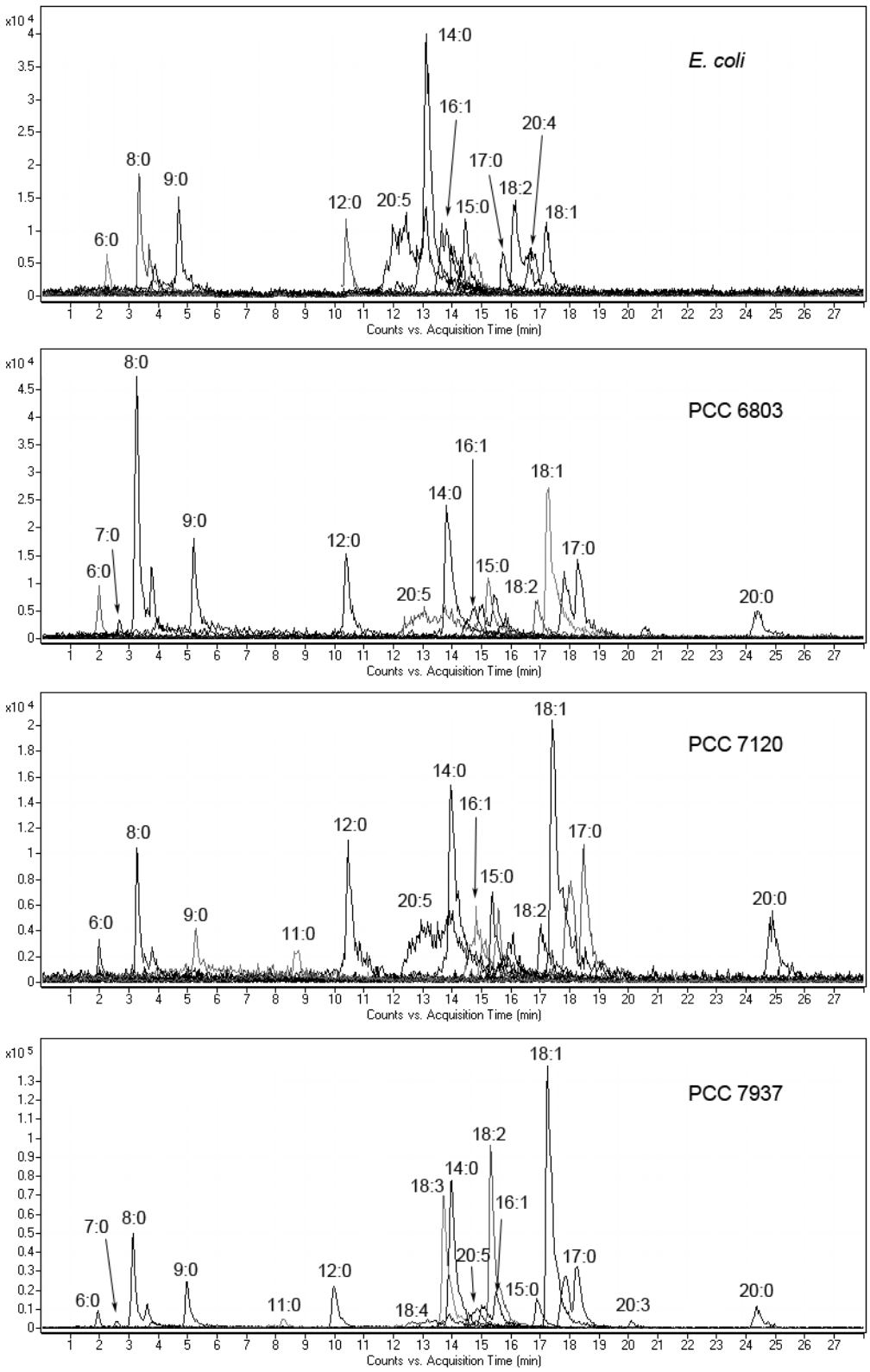


Figure 3.34. Extracted ion chromatograms of other fatty acids detected at lower abundance than in Fig. 3.33. Data was obtained from single QToF runs of spots that migrated to the solvent front of the aqueous phase, from extracts from *E. coli*, PCC 6803, PCC 7120 and PCC 7937.

Table 3.10. Integrated peak areas of other fatty acids detected at lower abundance in TLC scrapings from extracts of *E. coli*, PCC 6803, PCC 7120 and PCC 7937 from Fig 3.34. * = Odd chain length fatty acids could be due to methylated fatty acids, however this could not be confirmed using MS/MS.

Compound	**M – H m/z**	**Integrated peak areas**			
		E. coli	**PCC 6803**	**PCC 7120**	**PCC 7937**
6:0	115.076	37,350	84,255	20,635	81,466
7:0*	129.092	0	33,137	0	21,940
8:0	143.107	207,431	475,136	82,468	413,561
9:0*	157.123	141,797	165,275	27,651	245,255
11:0*	185.154	0	0	4,821	40,817
12:0	199.17	124,276	138,575	94,933	1,143,642
14:0	227.201	677,550	312,856	140,176	1,078,236
15:0*	241.217	72,117	97,261	39,657	130,434
16:1	253.217	64,789	13,698	11,493	78,324
17:0*	269.248	108,701	148,876	134,951	378,378
18:4	275.201	0	0	0	34,839
18:3	277.217	0	0	0	998,139
18:2	279.232	68,543	140,753	79,125	1,312,849
18:1	281.248	204,989	372,824	227,984	2,063,935
20:5	301.217	85,955	89,440	26,213	212,163
20:4	303.232	36,541	9,199	9,962	7,350
20:3	305.248	0	0	0	9,706
20:0	311.295	0	21,027	82,632	44,834

Table 3.11. Profile of complex lipids in *E. coli* that migrated on TLC plate where radioactivity was detected from 1-^{14}C-decanoic acid feeding.

Compound	Precursor ion m/z	Mass accuracy Δppm	Notes
PC(34:3)	756.553	1.0	Not possible to determine acyl chain composition from MS/MS data
PC(34:2)	758.5681	1.8	
PC(36:5)	780.5519	8.8	
PG(18:2/16:0)	745.4938	11.7	
PG(18:1/16:0)	747.5104	10.4	
PE(16:0/0:0)	452.2711	15.8	
PE(cyclo17:0/0:0)	464.2714	14.8	
PE(18:1/0:0)	478.2852	18.2	

Table 3.12. Profile of complex lipids in PCC 6803 that migrated on TLC plate where radioactivity was detected from 1-^{14}C-decanoic acid feeding.

Spots	Compound	Precursor ion m/z	Mass accuracy Δppm	Notes
Upper	DGDG(16:0/0:0)	653.3822	10.2	Formate adduct
Lower	SQDG(16:0/0:0)	555.2846	0.1	
	PC(34:3)	756.5338	26.0	Not possible to determine acyl chain composition from MS/MS data
	PC(34:2)	758.5493	26.5	
	PC(36:5)	780.5327	27.0	
	PC(36:4)	782.5467	29.0	
	PG(16:0/0:0)	483.2697	6.4	
	PG(18:2/0:0)	507.2749	4.1	

Table 3.13. Profile of complex lipids in PCC 7120 that migrated on TLC plate where radioactivity was detected from 1-^{14}C-decanoic acid feeding.

Spots	Compound	Precursor ion m/z	Mass accuracy Δppm	Notes
Upper	MGDG(18:3/16:0)	797.5467	5.8	As formate adduct
Lower	PC(34:3)	756.5338	2.5	Not possible to determine acyl chain composition from MS/MS data
	PC(34:2)	758.5493	40.3	
	PC(36:5)	780.5327	0.7	
	PC(36:4)	782.5467	0.5	
	PG(16:0/0:0)	483.2697	3.0	
	SQDG(34:1)	819.5253	5.5	Not possible to determine acyl chain composition from MS/MS data
	SQDG(34:2)	817.5169	3.3	
	SQDG(32:0)	793.518	4.8	
	SQDG(32:1)	791.5059	9.3	

Table 3.14. Profile of complex lipids in PCC 7937 that migrated on TLC plate where radioactivity was detected from 1-^{14}C-decanoic acid feeding.

Spots	Compound	Precursor ion m/z	Mass accuracy Δppm	Notes
Upper	MGDG(18:3/16:1)	795.5322	7.0	As formate adduct
Lower	DGDG(16:1/18:3)	957.5856	7.0	As formate adduct
	DGDG(16:0/18:4)	957.5856	7.0	
	DGDG(16:0/18:3)	959.6007	6.1	
	SQDG(30:0)	765.4887	7.7	Not possible to determine acyl chain composition from MS/MS data
	SQDG(32:2)	789.4820	1.0	
	SQDG(32:1)	791.4980	0.6	
	SQDG(32:0)	793.518	0.8	
	SQDG(33:0)	805.5192	6.3	
	SQDG(34:4)	813.4876	5.9	
	SQDG(34:3)	815.4891	0.5	
	SQDG(34:2)	817.5169	1.4	
	SQDG(34:1)	819.5253	5.5	
	SQDG(36:4)	841.5220	9.4	
	SQDG(36:3)	843.5343	5.4	
	SQDG(36:2)	845.5500	5.4	
	PG(16:0/16:0)	714.5104	32	

3.1.6. Discussion

β-oxidation is the major fatty acid degradative metabolic pathway in most organisms. However, these results provide some strong evidence for the apparent lack of such a pathway in cyanobacterial strains PCC 6803, PCC 7120 and PCC 7937.

Initial observations by bioinformatic analysis show that most β-oxidation genes annotated in the cyanobacteria are most likely misannotations. Acyl-CoA dehydrogenase-like enzymes have shown to be present in some strains of cyanobacteria, but it is more likely that these enzymes are non-ribosomal peptide synthetases, which have been associated with production of toxins such as microcystins, saxitoxins, anatoxin-a and nodularins in cyanobacteria (Neilan et al, 1999). Enzymes annotated as enoyl-CoA hyratases involved in β-oxidation have been shown to be other enzymes with somewhat similar catalytic activity; 6-oxocamphor hydrolase, β-diketone hydrolase and 1,4-dihydroxy-2-napthol-CoA synthase respectively. Enzymes annotated as 3-hydroxyacyl-CoA dehydrogenases appear to be 3-hydroxybutyryl-CoA dehydrogenases, and acyl-CoA acyltransferases appear to be PHA β-ketothiolases that are involved in PHA biosynthesis. PHAs are common energy storage compounds in bacteria (Byrom, 1987) and are used in the production of bioplastics.

The misannotation of protein sequences in public databases such as those highlighted in this study has been brought up before (Schnoes et al. 2009) and in order to definitively confirm or disprove the predictions from analysis of cyanobacterial genomes, it is necessary to provide real experimental evidence.

A starting point was to attempt to detect the initial substrate of this pathway, long-chain acyl-CoAs, in cyanobacterial extracts using a method that could show the presence of these molecules in a well characterized model organism, where it is known that these compounds exist – *E. coli* (Overath et al, 1969). This is the first time such a method

has been used to detect acyl-CoAs in microorganisms, though similar methods have been previously employed to determine acyl-CoAs in human blood (Veld et al. 2009), brain tissue (Deutsch et al. 2002) and in rat liver (Golokovo and Murphy, 2004; Mauriala et al. 2004). No acyl-CoAs could be found above the detection limit of 3 μM in any of the three SPE-enriched cyanobacterial extracts, with the exception of acetyl- and malonyl-CoA. Acetyl-CoA is a central metabolite and is involved in 38 different metabolic pathways according to the KEGG database, malonyl-CoA is involved in fatty acid biosynthesis, amino acid biosynthesis and the biosynthesis and metabolism of other secondary metabolites. While this assay was rapid and highly sensitive, a criticism of the assay could be in the minimal separation of compounds by retention time. Isomers could have the same m/z ratio but different structures, for example straight chain 17:0-CoA and methyl-branched 16:0-CoA have the same m/z, however they could be separated in the LC phase of the analysis as they interact with the stationary phase of the column differently, but due to the 3 minute run time minimal separation occurred between samples. Since it was purely detection of acyl-CoAs that was required and not specifically characterization, exact determination of the structures of acyl-CoAs was not critical to the overall aim of this experiment. Another possible issue with the short run time could be compromising of instrument sensitivity when attempting to analyze complex mixtures of acyl-CoAs such as those in extracts from cells; the relatively long scan time for each transition on the MS of 500 ms coupled with the short run time of the LC means that it was possible that some acyl-CoAs at low abundance in complex mixtures may have failed to be detected by the instrument, although in *E. coli*, a large range of acyl-CoAs were detectable.

Acyl-CoA dehydrogenase and acyl-CoA oxidase are the rate-limiting enzymes for β-oxidation (Aoyama et al. 1994; Kawaguchi et al. 1980). Acyl-CoA dehydrogenase is a flavoprotein that introduces a carbon-carbon double bond by transferring two hydrogens from carbons 2 and 3 on the acyl chain to the FAD prosthetic group, reducing it to $FADH_2$, which is then reoxidised back to FAD by coenzyme Q of the

electron transport chain (Thorpe and Kim, 1995; Beinert, 1962). Acyl-CoA oxidase has the same overall catalytic function, but it transfers the two hydrogens to molecular oxygen, forming peroxide, which is then converted into water by catalase that is present in the peroxisome Kim and Miura, 2004. No activity for either enzyme could be found in crude protein extracts from the four strains of cyanobacteria, yet activity for acyl-CoA dehydrogenase could be detected in *E. coli* and activity for acyl-CoA oxidase could be detected in cress seedlings.

Cyanobacteria when fed a labeled fatty acid, do not break it down as was observed in the heterotrophic *E. coli*, but utilize the fatty acid as a constituent of complex lipids, such as the glycolipids MGDG, DGDG and SQDG; and phospholipids PG, PE and PC. Glycolipids are structurally integral to the photosynthetic thylakoid membranes (Quinn and Williams, 1978), therefore it makes sense that cyanobacteria effectively utilize fatty acids for this purpose as they have many thylakoid membranes with in their cells.

These results open up a whole host of questions with regards to how cyanobacteria degrade fatty acids and lipids, or even if they do degrade them, strong evidence from Kaczmarkzyk and Fulda point to the possibility of fatty acid recycling in the form of re-ligating fatty acids back to ACP. The fatty acid biosynthetic pathway could be so tightly regulated in cyanobacteria that they produce just enough fatty acid needed for cell membranes; simple BLAST searching of *E. coli* fatty acid regulatory transcription factors FabR and FadR (Fujita et al. 2007) against cyanobacteria reveals that they do not possess significant alignments to these sequences, the interesting 2 exceptions to this rule are in the sequences of PCC 7425 and *A. marina*, which while not showing very high bit scores, are much higher than other cyanobacteria and E-values several orders of magnitude smaller. Another fate of synthesized fatty acids could be into the hydrocarbon heptadecane, which could be a metabolic end product.

It is still not known whether glycerol-3-phosphate acyltransferase and 1-acyl-sn-glycerol-3-phosphate acyltransferase use acyl-CoAs or acyl-ACPs for ligation of acyl

chains to glycerol backbones in the synthesis of triacylglyceride, phospholipids and glycolipids (Wilkison and Bell, 1997), as these enzymes have strong K_m values for both substrates. Our observations, along with those published by Kaczmarkzyk and Fulda are giving strong evidence as using acyl-ACPs as their main substrate, otherwise cyanobacteria would not be able to synthesise cell membranes.

Two exceptions to the observations in this chapter appear to be *Cyanothece* PCC 7425 and *A. marina* MBIC 11017, which are unusual strains. PCC 7425 has been observed to have unusual structural features, such as a high content of light refractile inclusions, concentric cortical thylakoids, a compact central nucleoid (Porta et al. 1999), as well as an unusual pattern of cell division compared to other cyanobacteria, and MBIC 11017 is unusual as it contains chlorophyll *d* (Marquardt et al. 1997), which uses far-red and near infrared light at 714 to 718 nm (Miyashita et al. 1996). A strain from the genus *Acaryochloris* is studied in Chapter 3.3.

The main issue with testing a hypothesis that states that a particular metabolic pathway is not present or is incomplete is that it could be there and proceeds by a novel route or by using novel compounds that current methods would not detect. For years it was accepted that cyanobacteria had an incomplete TCA cycle as they lacked 2-oxoglutarate dehydrogenase that converted 2-oxoglutarate to succinyl-CoA, however Zhang and Bryant reported 2 novel enzymes that performed the same function via a succinic semialdehyde intermediate, thereby completely bypassing the succinyl-CoA intermediate and forming succinate. So it is possible that fatty acids are broken down by another as yet unknown method, although it is clear from the radiolabelling carried out in this study that they do not end up as CO_2. Another noteworthy aspect of the above paper is that another coenzyme A thioester is not used in cyanobacteria as well as the lack of acyl-CoAs reported here. Evidence presented by Bennet et al. 2007, that PCC 7120 β-diketone hydrolase does not use a –CoA thioester substrate provides additional evidence for the lack of acyl-CoAs in cyanobacteria.

3.2 Ectopic expression of *E. coli* Acyl-CoA Synthetase in *Synechocystis* PCC 6803

3.2.1 Introduction

In chapter 3.1 it was demonstrated that there was evidence to show that β-oxidation of fatty acids does not occur in cyanobacterial strains PCC 6803, PCC 7120 and PCC 7937. In this chapter an attempt was made to introduce an element of the β-oxidation pathway into PCC 6803 in the form of acyl-coA synthetase from *E. coli*. As discussed in 1.4, in bacteria this enzyme is responsible for activating fatty acids by ligating them to coenzyme A to form acyl-CoAs, which can then enter the β-oxidation pathway by being dehydrogenated by acyl-CoA dehydrogenase and further oxidized by other enzymes of the β-oxidation pathway to ultimately form acetyl-CoA. The ligation proceeds via a two-step mechanism involving the hydrolysis of ATP to pyrophosphate to drive the endergonic reaction, forming a fatty acyl adenylate intermediate, the carboxyl group of which undergoes nucleophilic attack from the thiol sulphur of CoA-SH, forming the end product acyl-CoA plus AMP and pyrophosphate. *E. coli* was chosen as a "donor" for its' acyl-CoA synthetase gene, *fadD* as it is easy to grow, extract DNA from and perform general molecular biology on; it only has one acyl-CoA synthetase gene (Kameda et al. 1985) as opposed to other organisms such as humans (7), *A. thaliana* (11), *B. subtilis* (2), *S. cerevisiae* (4) or *C. reinhardtii* (4), furthermore this enzyme has broad chain length specificity (Kameda and Nunn, 1981) and so can utilize any free fatty acids as substrate that may be present within the cellular pool of PCC 6803.

Upon successful introduction of this enzymes' coding gene, *fadD*, into PCC 6803, the strain was tested to determine if 1. It is expressed, using RT-PCR; 2. It is functional, i.e. can synthesise acyl-coAs, using the acyl-CoA assay described in 3.2.3, and by an enzymatic activity assay utilizing ^{14}C-labeled fatty acids, described in this chapter.

Generation of *fadD*-expressing PCC 6803 was achieved using a semi-synthetic biology approach, utilizing BioBrick methodology (Shetty, 2008). Much of the molecular biology described in this chapter was based on the tools developed by Huang et al. 2010.

3.2.2 Amplification of elements to be inserted into expression vector.

The promoter sequence derived from the large subunit of Rubisco, *PrbcL* was amplified from PCC 6803 genomic DNA using the primers and PCR conditions described in 2.16.4. *fadD* from *E. coli* DH5α genomic DNA (gDNA) was amplified using the primers and PCR conditions described in 2.16.3. Both sets of conditions were arrived at after optimization of annealing temperature of primers to template (Fig. 3.35). Additionally *PrbcL* required extra optimization, as initially very little PCR product was detectable. In order to determine whether this was due to primers or genomic DNA preparations, a control PCR was carried out using primer sequences Fwd: AGCGGTGTAATTTCGTCACC & Rev: GCCGATATGGTGAGGTCCTA that amplified a 185 bp region within the DNA sequence of the *rpoD* gene, an RNA polymerase sigma factor that was known previously to give a PCR product, so if no PCR product was detected then the gDNA preparation was the cause, but a PCR product was detected using agarose gel electrophoresis (Fig. 3.36, A) so it was determined that the primer was the cause. Purification of the primers using the method described in 2.14.4, along with the use of 1.25 % DMSO to reduce the possibility of secondary structure formation in the DNA template (fig 3.36, B) resulted in successful amplification of the 254 bp PCR product (fig 3.36, C).

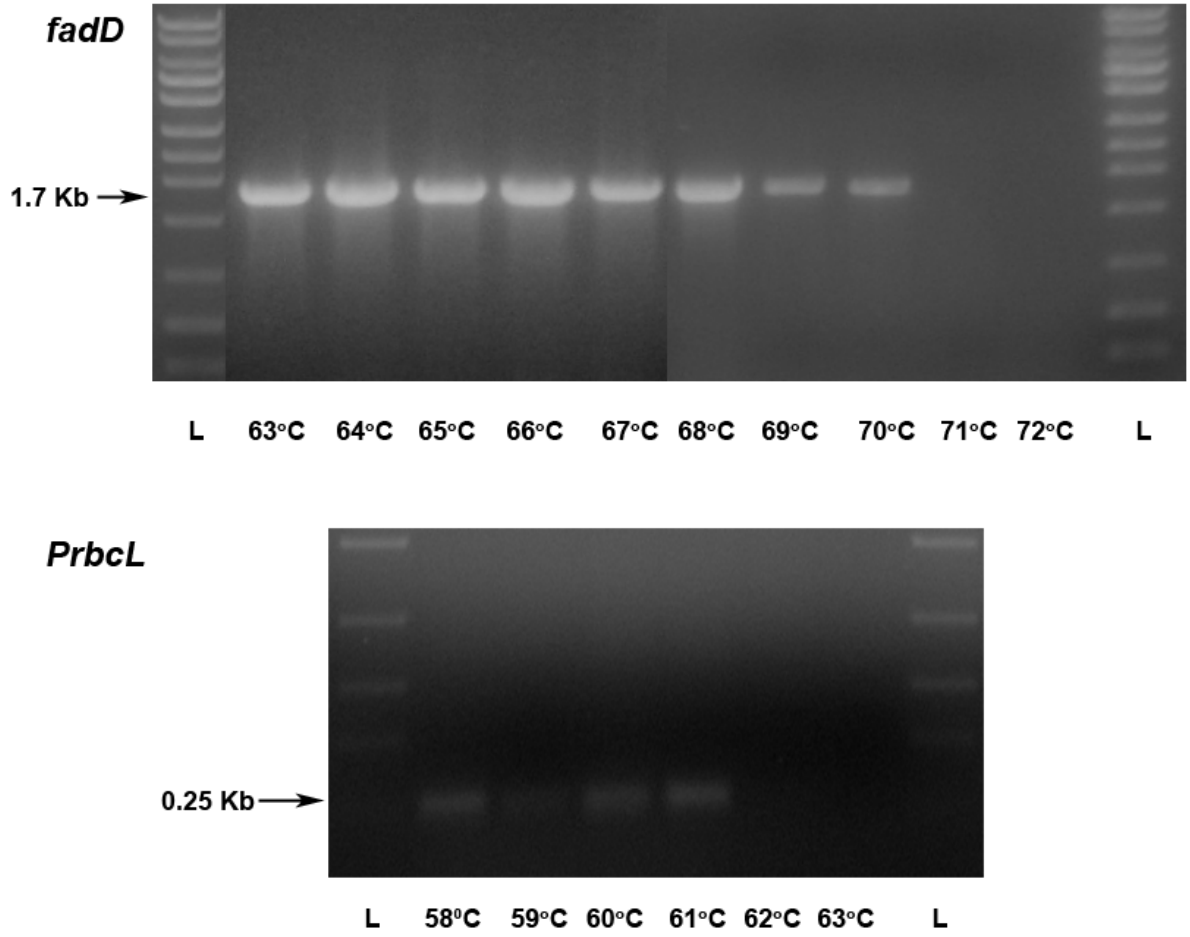


Figure 3.35. Optimisation of annealing temperatures for *fadD* primers and *PrbcL* primers.

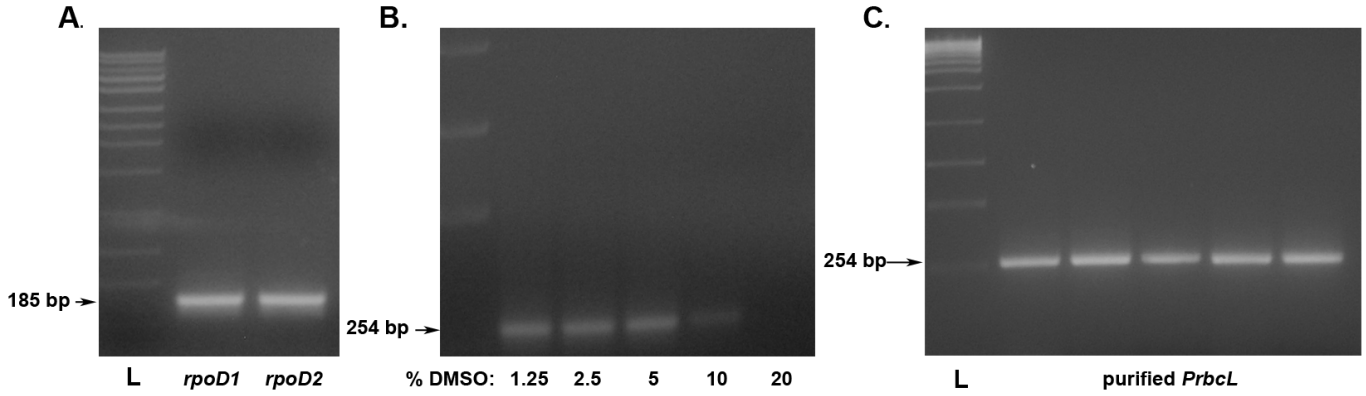


Figure 3.36. Optimisation of PCR of the *rbcL* promoter from PCC 6803. **A.** Control PCR of 185 bp *rpoD* fragment. **B.** 1.25 % DMSO improves efficiency of PCR of *PrbcL*. **C.** PCR of *PrbcL* from 5 PCC 6803 gDNA preparations using purified primers.

3.2.3 Preparation of BioBrick vectors

PCR products *fadD* and *PrbcL* from 3.3.2 were flanked with BB sites that contain the restriction sites EcoRI and XbaI at the 5' end of the sequence and PstI and SpeI at the 3' end of the sequence. These BB sites were digested with restriction endonucleases EcoRI and PstI. At the same time, BioBrick cloning vectors pSB1A3 and pSB1C3 that were shipped containing BioBrick part: BBa_J04450, a gene encoding for the production of red fluorescent protein (RFP), were digested with EcoRI and PstI, successfully excising the RFP fragment from the vector as can be seen in Fig. 3.37, A, with a band present at approximately 1.2 Kb corresponding with the size of the RFP fragment, and the cut, linearised vector at approximately 2.2 Kb and 2.1 Kb for pSB1A3 and pSB1C3 respectively. *fadD* was ligated into pSB1A3 and *PrbcL* was ligated into pSB1C3 and transformed into *E. coli* strain TOP10 and plated with appropriate antibiotic selection. The fact that pSBx vectors were shipped with the RFP gene was useful for quick determination of whether the restriction digests were successful, as colonies don't have the desired PCR products inserted and still contain RFP fluoresce red (Fig. 3.37, B).

For definite confirmation of insertion of *fadD* and *PrbcL* into their respective pSBx vectors, colony PCR was performed using verification primers (Fig. 3.38 and 3.39), showing that all 5 replicates of the pSB1A3 ligation contained *fadD*, producing a band at c. 2 Kb, primer binding sites on the vector are about 150 bp upstream and downstream of the BB site where the 1.7 Kb *fadD* PCR product was inserted. Colony PCR was performed on 14 replicates of the pSB1C3 vector containing *PrbcL*, 7 gave a band of the correct size of c. 590 bp (Fig. 3.39, B), due to primer binding sites being c. 170 bp upstream and downstream of the BB site where the 254 bp *PrbcL* sequence inserted. In some samples a 340 bp PCR product was observed due to ampification of empty vector that contained no fragment in the BB site (Fig. 3.39, B). Additionally colony PCR was performed on pSB1C3 using the primers used to amplify *PrbcL* from PCC 6803 gDNA, resulting in 10 of the 14 samples showing bands on the gel, indicating that these extra 3 bands are false positives (Fig. 3.39, A). Note that colony PCR could not be performed on pSB1A3 using *fadD* amplification primers, as endogenous *fadD* amplification from *E. coli* TOP10 chromosomal DNA would have given false positive results.

Additional confirmation of insertion of *fadD and PrbcL* into vectors was sought by performing diagnostic restriction digests with NotI and comparing to in-silico predictions of how vectors digest (Fig. 3.40). pSB1A3 containing *fadD* and pSB1C3 containing *PrbcL* digested as expected, bands at 2131 bp and 1711 bp for pSB1A3 and 2046 bp and 296 bp for pSB1C3.

In order to show no mutations had arisen that affect the coding sequence of *fadD* or affect the binding of cyanobacterial RNA polymerases to the *PrbcL* sequence; it was necessary to sequence the vectors. No mutations were detected in the forward or reverse sequences of the *fadD* insert in pSB1A3 (Fig. 3.42) using the VF2 and VR primers. 1 point mutation was detected in the *PrbcL* sequence, a nucleotide substitution from a guanine in the *in-silico* sequence to a cytosine in the sequenced vector (Fig. 3.41).

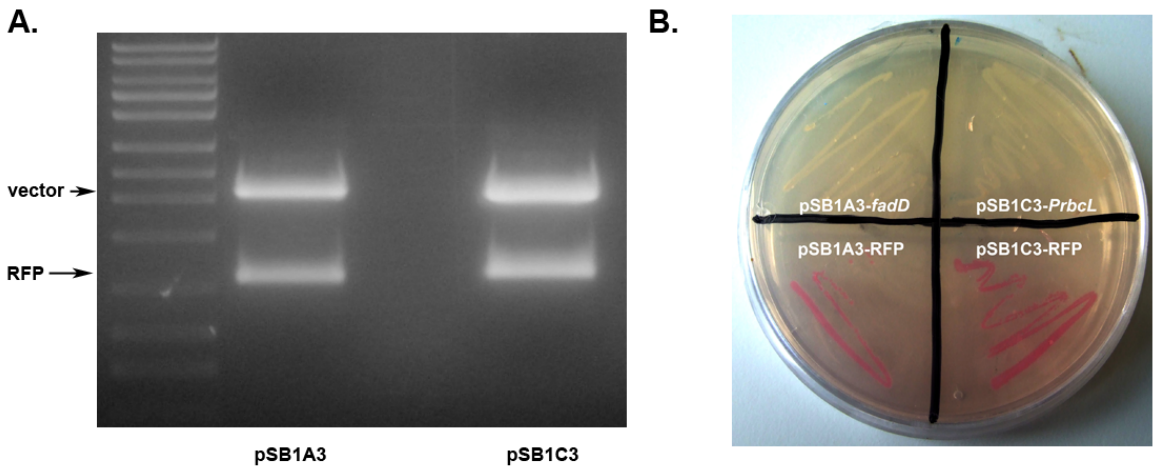


Figure 3.37. Successful excision of RFP from pSBx cloning vectors. **A.** Agarose gel of restriction digests of pSBx cloning vectors. **B.** RFP-containing vectors cause hosts to appear red.

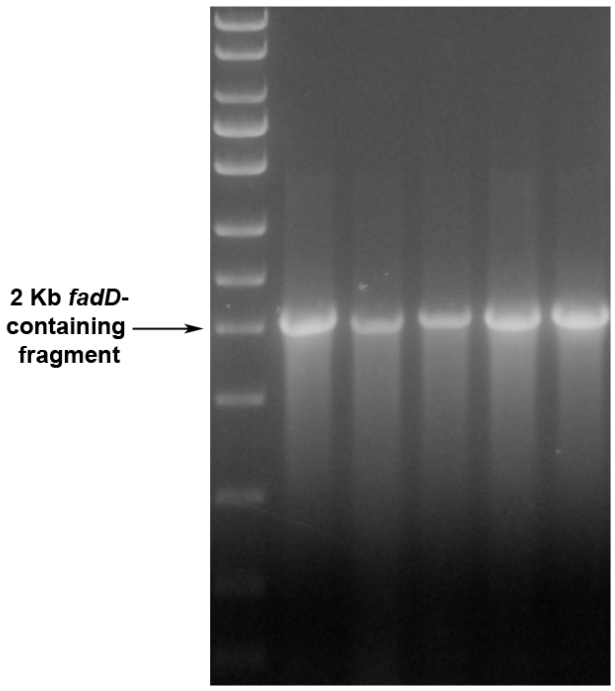


Figure 3.38. Colony PCR of *fadD*-containing fragment from 5 TOP10 colonies transformed with pSB1A3, showing ampification of 2 Kb *fadD* containing PCR product.

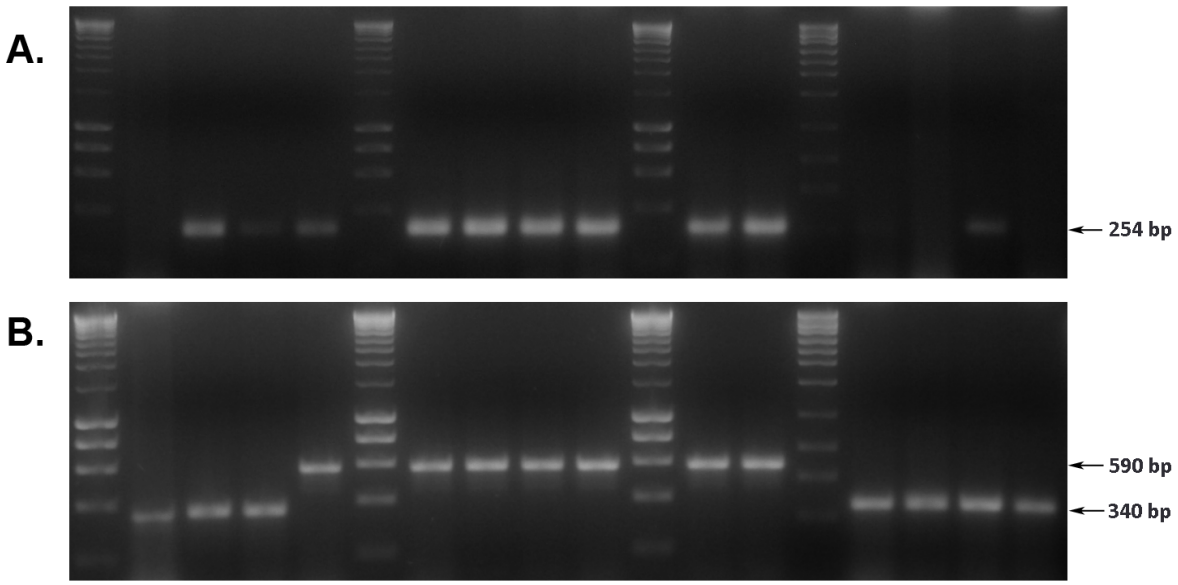


Figure 3.39. PCR of *PrbcL*. **A.** Colony PCR of *PrbcL* from *E.coli* transformed with pSB1C3, with primers used to initially amplify from PCC 6803 gDNA, showing product at 254 bp. **B.** Colony PCR of *PrbcL* using verification primers. Correct *PrbcL* containing PCR product at 590 bp. Incorrect PCR product at approx. 340 bp.

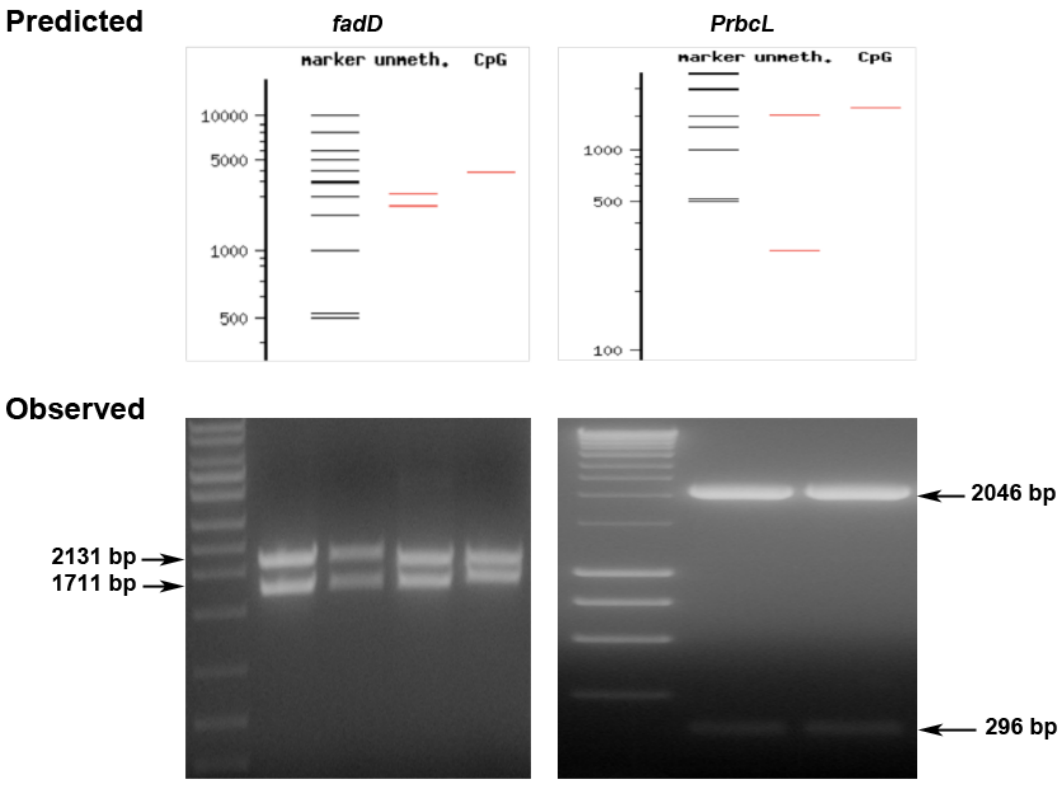


Figure 3.40. *In-silico* predicitons and observed *in-vitro* results of diagnostic restriction digests of pSBx vectors using NotI. pSBx vectors containing *fadD* and *PrbcL* inserts digested *in-vitro* as was predicted *in-silico*, indicating that vectors contained their desired inserts.

```
PrbcL_seq      AGAGTCACCATTTGGACAAAACATCAGCAATTCTAATTAGAAAGTCCAAAAATTGTAATT
rbcL promoter  ---------------GACAAAACATCAGGAATTCTAATTAGAAAGTCCAAAAATTGTAATT

PrbcL_seq      TAAAAAACAGTCAATGGAGAGCATTGCCATAAGTAAAGGCATCCCCTGCGTGATAAGATT
rbcL promoter  TAAAAAACAGTCAATGGAGAGCATTGCCATAAGTAAAGGCATCCCCTGCGTGATAAGATT

PrbcL_seq      ACCTTCAGAAAACAGATAGTTGCTGGGTTATCGCAGATTTTTCTCGCAACCAAATAACTG
rbcL promoter  ACCTTCAGAAAACAGATAGTTGCTGGGTTATCGCAGATTTTTCTCGCAACCAAATAACTG

PrbcL_seq      TAAATAATAACTGTCTCTGGGGCGACGGTAGGCTTTATATTGCCAAATTTCGCCCGTGGG
rbcL promoter  TAAATAATAACTGTCTCTGGGGCGACGGTAGGCTTTATATTGCCAAATTTCGCCCGTGGG

PrbcL_seq      AGAAAGCTAGGCTATTCAATGTTTAAAGAGGAGAAATA
rbcL promoter  AGAAAGCTAGGCTATTCAATGTTT--------------
```

Figure 3.41. Alignment of sequenced pSB1C3 containing *PrbcL* insert (PrbcL_seq) vs. *in-silico* predicted sequence (rbcL promoter). Mismatches are highlighted in red.

A.

B.

Figure 3.42. Alignment of sequenced pSB1A3 containing *fadD* insert vs. *in-silico* predicted sequence. **A.** Forward sequence. **B.** Reverse sequence. Mismatches are highlighted in red.

3.2.4 BioBrick assembly

PrbcL and *fadD* contained in plasmids pSB1C3 and pSB1A3 respectively were assembled into destination plasmid pPMQAK1 as described in 2.14.12. Before carrying out the assembly, it was confirmed that pPMQAK1 digested as expected using EcoRI and PstI (Fig. 3.43), giving fragments of 7.7 Kb and 705 bp.

TOP10 colonies transformed with *PrbcL-fadD* containing pPMQAK1 were verified to have the correct inserts present by colony PCR (Fig. 3.44), 5 out of the 10 colonies assessed showed a PCR product at the correct size of approximately 2.3 Kb, accounting for the 1.7 Kb *fadD* gene, 254 bp *rbcL* promoter, as well as 151 bp upstream and 180 bp downstream of the insert to the primer binding sites respectively, adding up to a total fragment size of 2271 bp. Diagnostic restriction digests of pPMQAK1 DNA from these 5 colonies using ScaI also gave bands of the expected size as the *in-silico* predictions of 5.9 Kb and 3.8 Kb (Fig. 3.45). TOP10 colonies that were transformed with either undigested pPMQAK1 containing *ccdB* would not have grown as this gene is toxic, and colonies that were transformed with any pSBx plasmid would not have grown as they did not have the the kanamycin resistance cassette that pPMQAK1 was carrying.

To ensure that no mutations had been introduced into *PrbcL* and *fadD*, sequencing was carried out as described in 3.2.3. However due to the 2.3 Kb size of the insert, and that sequencing reads give reliable basecalls for a maximum of about 1 Kb, it was necessary to utilize a third priming site 317 bp into the *fadD* gene in order to give complete sequencing coverage of the insert. The only mutation present was the one observed in *PrbcL* that had carried over and is described in 3.2.3 (Fig. 3.46).

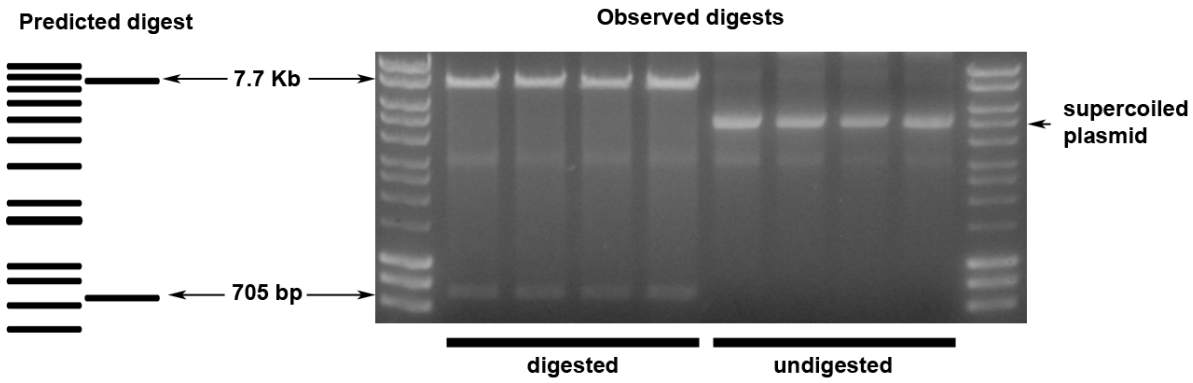


Figure 3.43. Restriction digest of *ccdB*-containing pPMQAK1 with EcoRI and PstI. *In-vitro* diagnostic digest matched up with *in-silico* prediction, confirming that pPMQAK1 digests as expected.

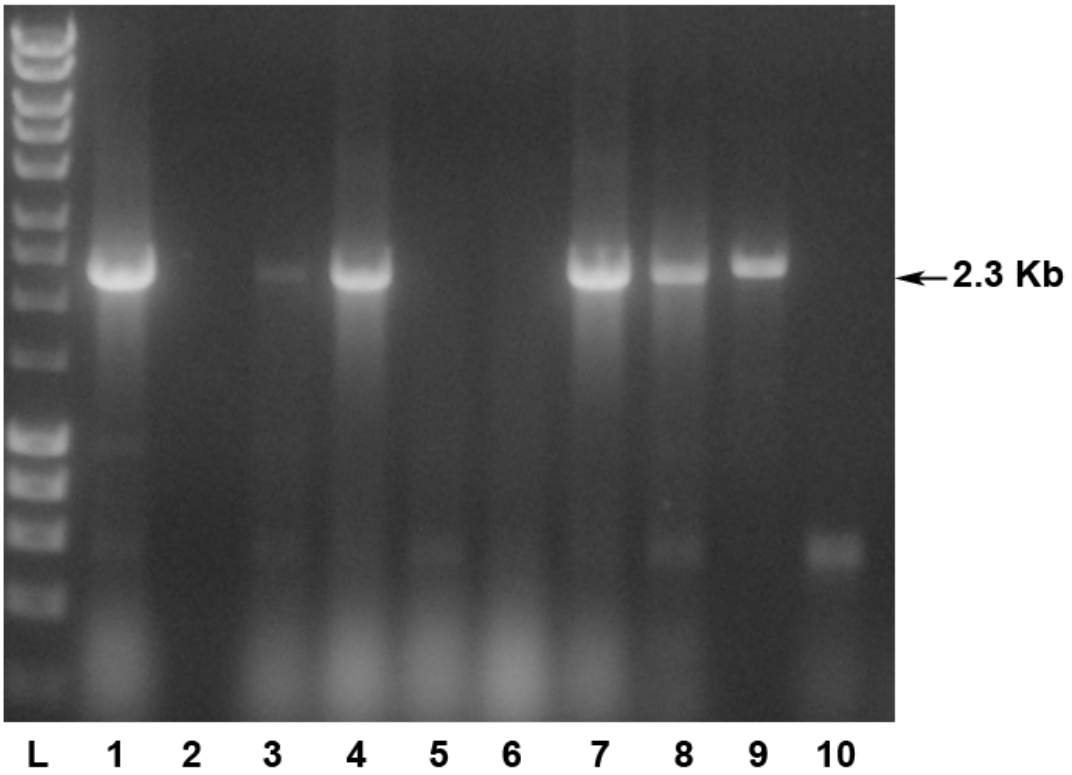


Figure 3.44. Colony PCR of *PrbcL-fadD* insert from pPMQAK1 using verfication primers, showing *PrbcL-fadD*-containing fragment at 2.3 Kb.

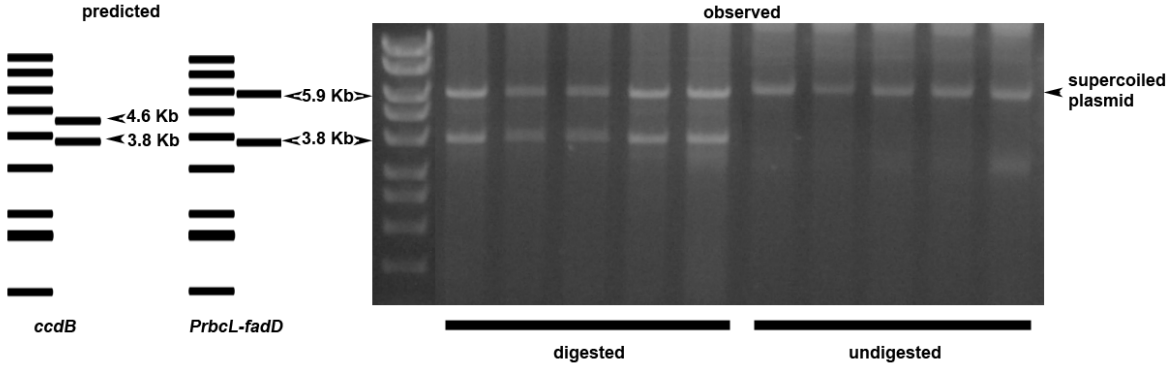
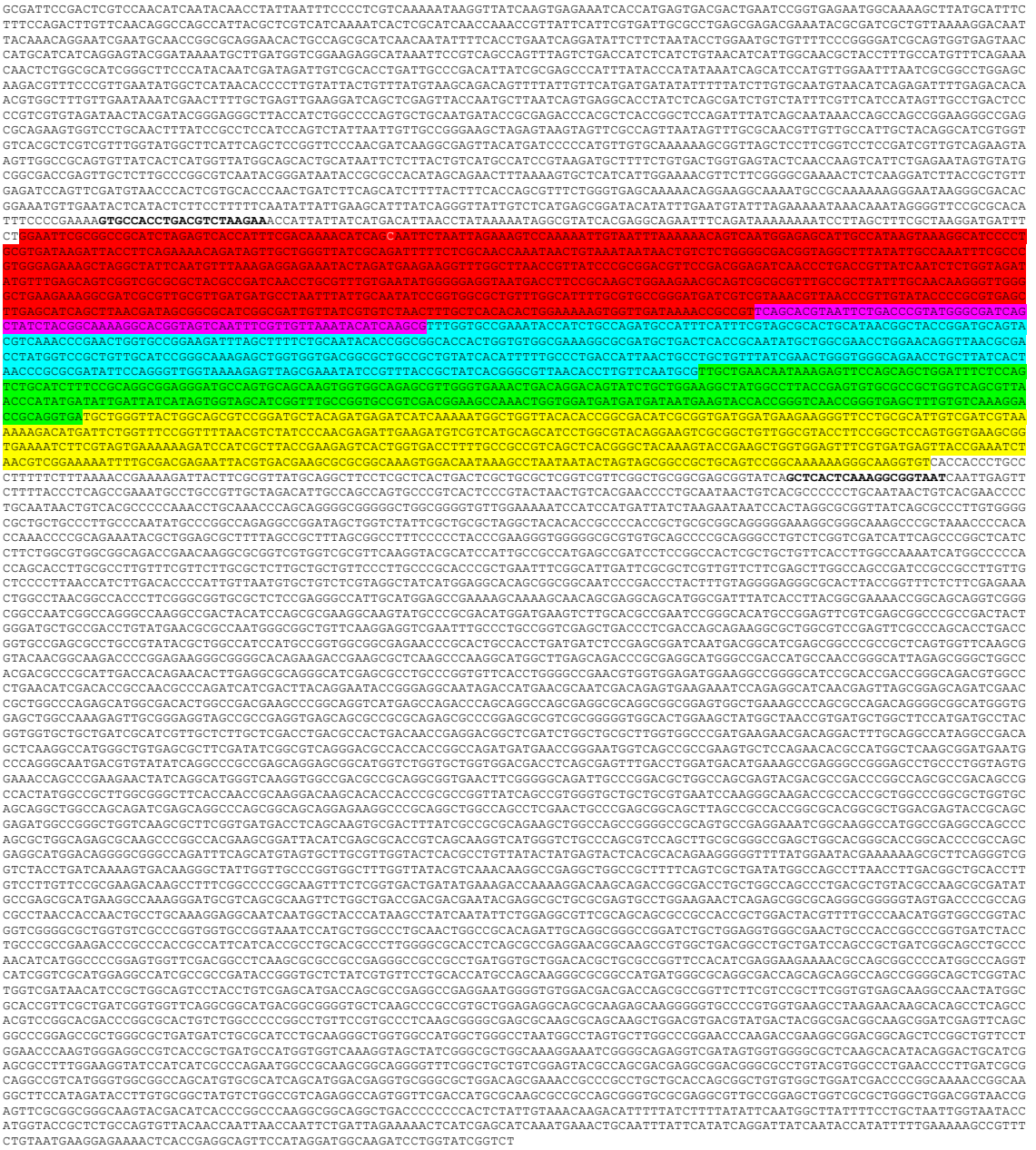


Figure 3.45. Restriction digests of pPMQAK1 containing *PrbcL* and *fadD* using ScaI. *In-vitro* digested pPMQAK1-*PrbcL-fadD* digested as was predicted in-silico for all 5 replicates. No DNA fragments were observed for the *in-silico* predicted digest of *ccdB*-containing pPMQAK1 plasmid by ScaI.



Key:

Forward verification primer Middle verification primer

Reverse verification primer Mixed forward and middle Mixed middle and reverse

Figure 3.46. DNA sequence of pPMQAK1 containing *PrbcL* and *fadD*. Sequencing priming sites are highlighted in bold. Mismatch (G to C) highlighted in white. Highlighted regions show where plasmid was sequenced using specific primers.

3.2.5 Transformation of PCC 6803

Colonies of PCC 6803 transformants were observed to grow as single colonies after a period of dieback from non-transformed colonies, lasting over a period of 28 days (Fig. 3.47). Upon transfer and growth of these transformants in liquid BG-11 containing 50 $\mu g\ ml^{-1}$ kanamycin, it was found by streaking samples of the PCC 6803 transformants onto LB-agar plates and incubating in the dark at 37 °C for 24 h and so favouring the growth of any contaminating heterotrophic bacteria, that these cultures were still heavily contaminated with the *E. coli* (Fig. 3.48). After replating PCC 6803 colonies, low level contamination was still present, as no growth was visible after plating on LB-agar after 24 h (Fig. 3.50), but was visible after a period of 1 week (168 h).

Bacteriophage T4 was employed to remove any remaining contaminating *E. coli* by lysing the cells. The phage was first tested on cultures of *E. coli* DH10B carrying the pPMQAK1 plasmid containing *PrbcL* and *fadD*, added in early log phase of growth and was found to effectively halt the growth of *E. coli* cultures compared to control and optical density was observed to slowly fall due to cell lysis (Figure 3.49). Applying T4 bacteriophage at the same concentration to cyanobacterial cultures reduced contamination effectively to zero, as can be seen in Fig 3.50 for cultures B and D that were treated with phage, with no *E. coli* growth present, whereas low level growth was showing after 72 h. In order to further confirm this, a gene that is found only in *E. coli, fadR,* was amplified from the replated transformants (Fig 3.51, A) and compared to a PCC 6803 culture that was known to be contaminated with *E. coli*, (Fig 3.51, C) this level of contamination was extremely low and was probably only visible due to the high sensitivity of PCR.

It was confirmed that transformants contain the BioBrick insert containing the *fadD* gene and the *rbcL* promoter by PCR (Fig. 3.52). *fadD* alone was amplified from plasmid DNA using the primers that amplified *fadD* from *E. coli* genomic DNA initially, and whole BioBrick inserts were amplified using the verification primers (Fig. 3.52). While all 6 of the 1.7 Kb *fadD* sites were successfully amplified, only 4 of the BioBrick sites were successfully amplified. Also present was evidence of possible empty vectors or vectors containing the *rbcL* insert only, as smaller PCR products were also present. It is possible that *fadD* was able to be amplified from all replicates of the transformants due to the low level contamination of *E. coli* remaining from the transformation step, thus giving false positive results. Therefore only the 4 transformants giving PCR products using the verification primers were used in later experiments.

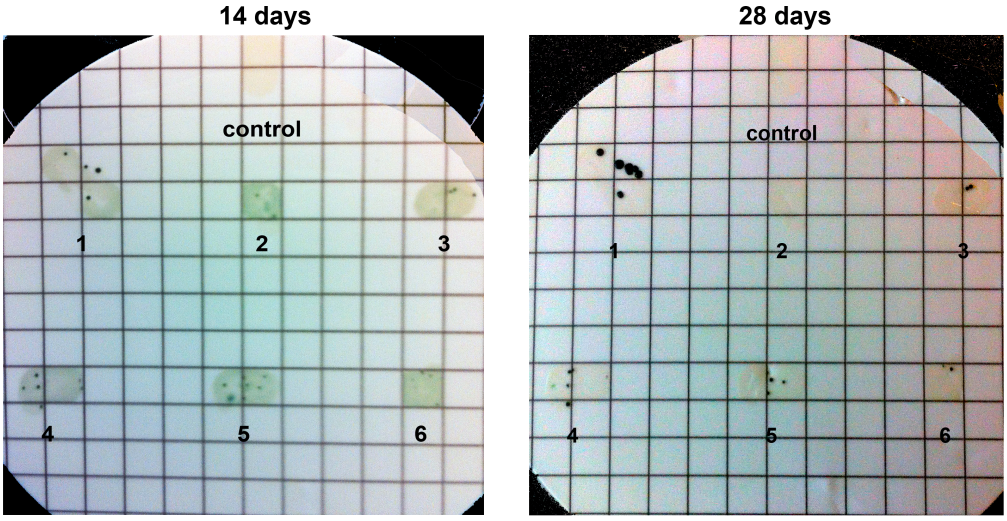


Figure 3.47. Growth of PCC 6803 colonies on nitrocellulose discs on BG11 agar medium containing 50 µg ml^{-1} after spot mating at 14 days and 28 days. Untransformed control colony dies back rapidly. First colonies start to appear after 14 days and untransformed cyanobacteria have completely died off after 28 days.

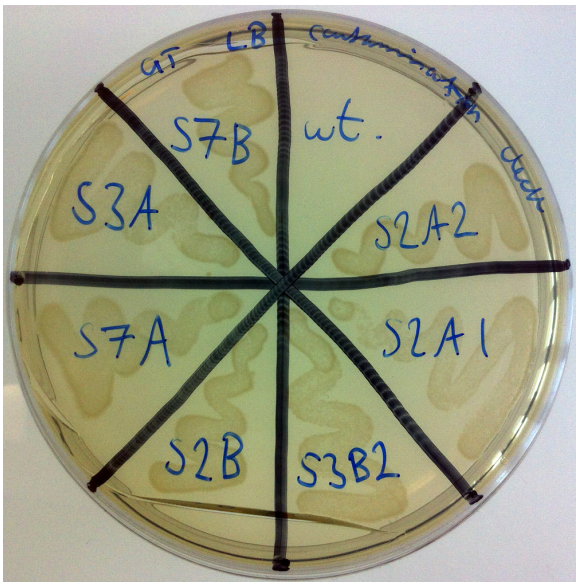


Figure 3.48. Contamination monitoring of PCC 6803 transformants by streaking on LB-agar plates. Growth of *E. coli* is visible from 7 cultures of PCC 6803 transformants and no growth from an uncontaminated wild-type culture.

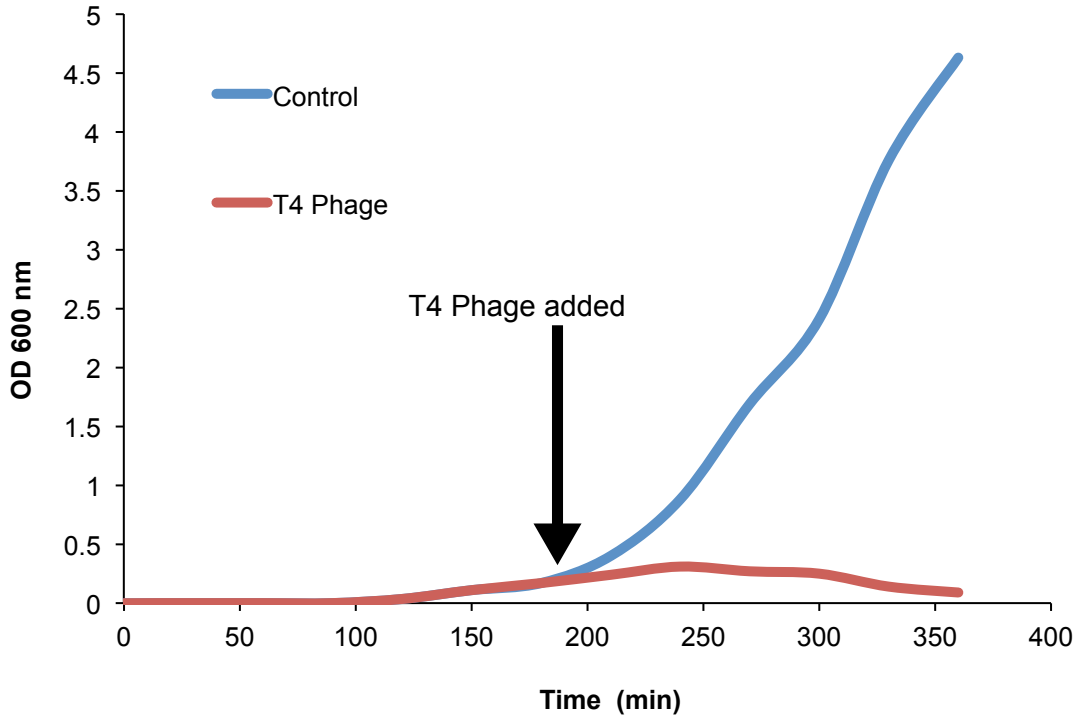
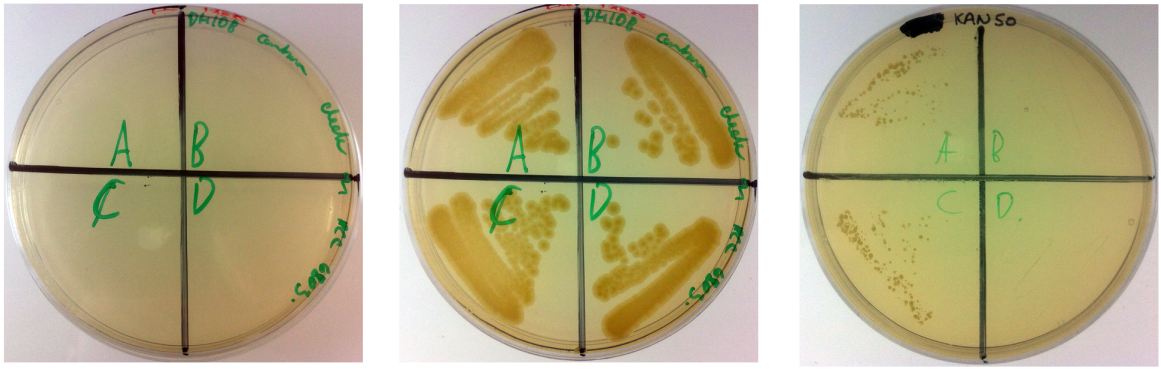


Figure 3.49. Growth curve of *E. coli* DH10B carrying the pPMQAK1 plasmid containing *PrbcL* and *fadD*, both with and without bacteriophage T4 present in cultures. Experiment was conducted over a course of 360 min, as quantified by measurement of $OD_{600\ nm}$.



24 h **168 h** **72 h after phage**

Figure 3.50. Contamination monitoring of PCC 6803 transformants by streaking on LB agar plates. Left: Contamination was reduced by restreaking PCC 6803 transformants, so no *E coli* growth was observed after 24 h. Middle: Low level contamination was still present, as growth was observed after 1 week. Right: Cultures B and D were treated with phage and low level contamination appeared to be removed, compare to cultures A and C where contaminating *E. coli* growth can be observed.

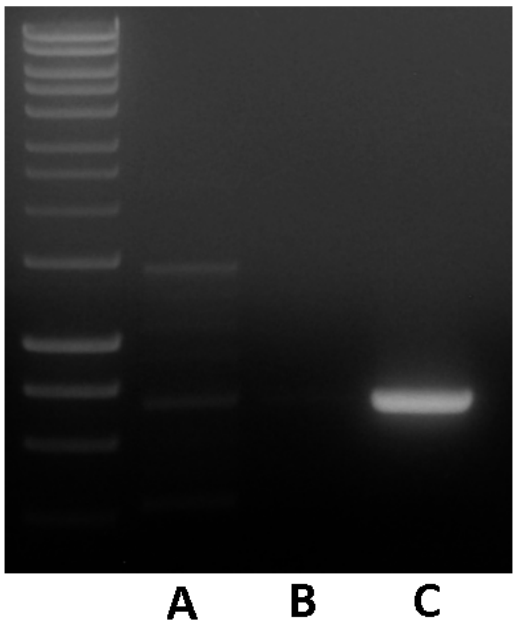


Figure 3.51. 750 bp *fadR* endogenous to *E. coli* only was amplified from total DNA of PCC 6803 transformants that had been purified by replating and incubation with T4 phage (**A**); wild-type PCC 6803 (**B**); and PCC 6803 that was known to be contaminated with *E. coli* (**C**).

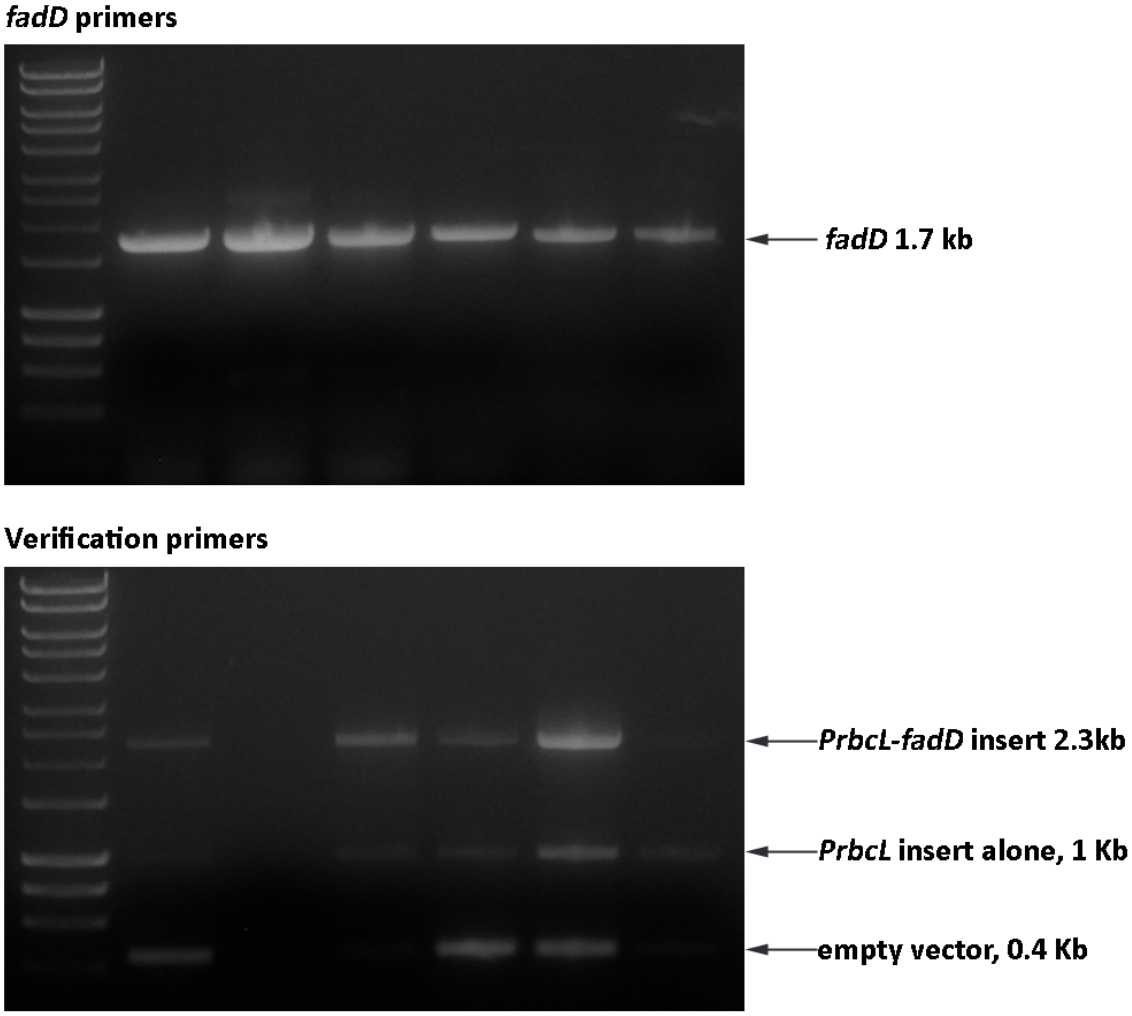


Figure 3.52. PCR results from plasmid DNA extracted from PCC 6803 transformed with pPMQAK1 containing *PrbcL* and *fadD*. *fadD* alone could be amplified from all transformants, but the full *Prbc-fadD* insert could only be amplified from 4 of the 6 transformants.

3.2.6. Analysis of transformants

In order to determine whether the successfully transformed *fadD* gene was transcribed into an mRNA message, RNA was extracted from the cyanobacteria, converted into cDNA and PCR using *fadD* primers was performed.

Expression of *fadD* appeared to be very high as can be seen in the gel picture (Fig. 3.53), compare to the bands in the DNA ladder at 10 Kb and 1 Kb that have 100 ng of DNA present. In order to quantitatively demonstrate that this gene was expressed at a significantly higher level than other genes, RT-PCR would have been performed on a range of control genes in PCC 6803 for comparison, however the purpose of this experiment was essentially a qualitative one, to determine whether the gene was transcribed at all. As a control RNA sample that was not reverse transcribed was not ran on the gel, there is the possibilty that contamininating genomic DNA could have interfered with the results in Fig 3.53.

As would be expected, the gene appears to be expressed at a high level because it is under control of the *rbcL* promoter, encoding for the large subunit of RuBisCO that is expressed at a high level in photosynthetic organisms (Ellis, 1979), and accounts for up to 50 % of total protein in some plant species (Feller et al. 2008).

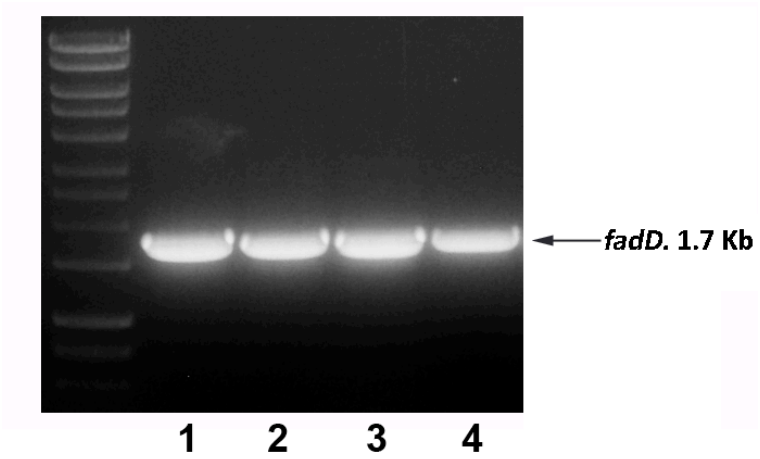


Figure 3.53. PCR of *fadD* taken from cDNA preparations of PCC 6803 transformants; agarose gel image of *fadD* cDNA transcripts, amplified DNA fragments at 1.7 Kb.

Acyl-CoAs can be detected in extracts of PCC 6803 *fadD* transformants. Each of the four replicates of PCC 6803 expressing *E. coli fadD* were pooled, harvested and extracted for acyl-CoAs as described in 2.5. Initially no acyl-CoAs could be detected, probably because there was a limited pool of free fatty acid substrate. Upon feeding the cyanobacteria 0.3 mM palmitic acid for 24 h before extraction, six different long chain fatty acids (18:0-CoA, 16:0-CoA, 14:0-CoA, 18:3-CoA, 18:2-CoA and 16:1-CoA) and one short chain fatty acid, butanoyl-CoA (4:0-CoA, also known as butyryl-CoA) were detectable (Fig. 3.54) in addition to acetyl-CoA and malonyl-CoA that were also detected in wild-type PCC 6803 extracts (Fig. 3.11).

16:0-CoA was the most abundant acyl-CoA (Table 3.15), probably because this is the CoA derivative of palmitic acid that was fed to the culture.

Possible explanations for presence of other long chain fatty acids detected in extracts could be that these were low level contaminants in the palmitic acid preparation, and some of these fatty acids such as 14:0, 16:1, 18:2, 18:3 and 4:0 are more soluble in water than palmitic acid, so more of this compound was available to the cyanobacteria for uptake. Another explanation could be that some of these compounds may have a greater ionization efficiency in the ESI of the mass spectrometer than 16:0-CoA and so

a larger signal intensity would be observed, even if there were less of this compound present in extracts. Authentic standards could be used to obtain absolute quantitation, however none was commercially available at the time of performing these experiments. Another less likely explanation for the presence of 16:1-CoA, 18:0-CoA, 18:2-CoA and 18:3-CoA could be that 16:0-CoA was either being extended and/or desaturated by acyl-ACP -accepting fatty acid biosynthesis enzymes and desaturated by desaturase enzymes (Los et al. 1997), as it is possible that these two enzymes may have some cross-reactivity with coenzyme A thioesters and be able to accept an acyl-CoA substrate in addition to their usual acyl-ACP substrates, as has been observed in glycerol-3-phosphate acyltransferases in *E. coli* (Wilkison and Bell, 1997).

The presence of butanoyl-CoA was also somewhat puzzling, as activity from acyl-CoA synthetase on chain lengths as low as 4:0 have not been reported previously, the lowest chain length being hexanoic acid (Kameda and Nunn, 1981), although this could be simply because they had not looked at the enzymes' activity on butanonic acid rather than not being able to detect it. 4:0-CoA was also detectable in extracts from *E. coli* (Fig. 3.9), but it was assumed that this was a metabolic intermediate of the β-oxidation pathway. The fact that 4:0-CoA can be detected in PCC 6803 that does not posess the β-oxidation pathway raises further questions as to where this butanoic acid substrate came from. Factoring in that in *fadD*-expressing PCC 6803 extracts that were not fed any fatty acid, nothing could be detected besides acetyl-CoA and malonyl-CoA, it could be assumed that butanoic acid was also a contaminant found in the palmitic acid preparation, although the palmitic acid preparation was certified >99 %.

The possibility of contamination from *E. coli* can be ruled out, as these cultures have been shown to have negligible levels of contamination in 3.2.5. Furthermore, the profiles of acyl-CoAs are different, with *E. coli* possessing no 18:2-CoA or 18:3-CoA and different relative amounts other acyl-CoAs (Table 3.15), although this change

could be caused by the different environmental conditions that PCC 6803 are grown in, however these growth conditions would be very difficult to replicate.

Table 3.15. Relative peak areas and percentage of total peak areas of each acyl-CoA that were detected in PCC 6803 *fadD* transformants.

Acyl-CoA	**Peak area in PCC 6803 *fadD***	**% total in PCC 6803 *fadD***	**% total in *E coli***
4:0-CoA	151	6.2	5.2
malonyl-CoA	174	7.2	25.3
acetyl-CoA	458	18.9	45.5
18:0-CoA	108	4.4	0.1
16:0-CoA	591	24.3	7.0
14:0-CoA	38	1.6	6.5
18:3-CoA	378	15.6	0
18:2-CoA	304	12.5	0
16:1-CoA	227	9.3	1.2

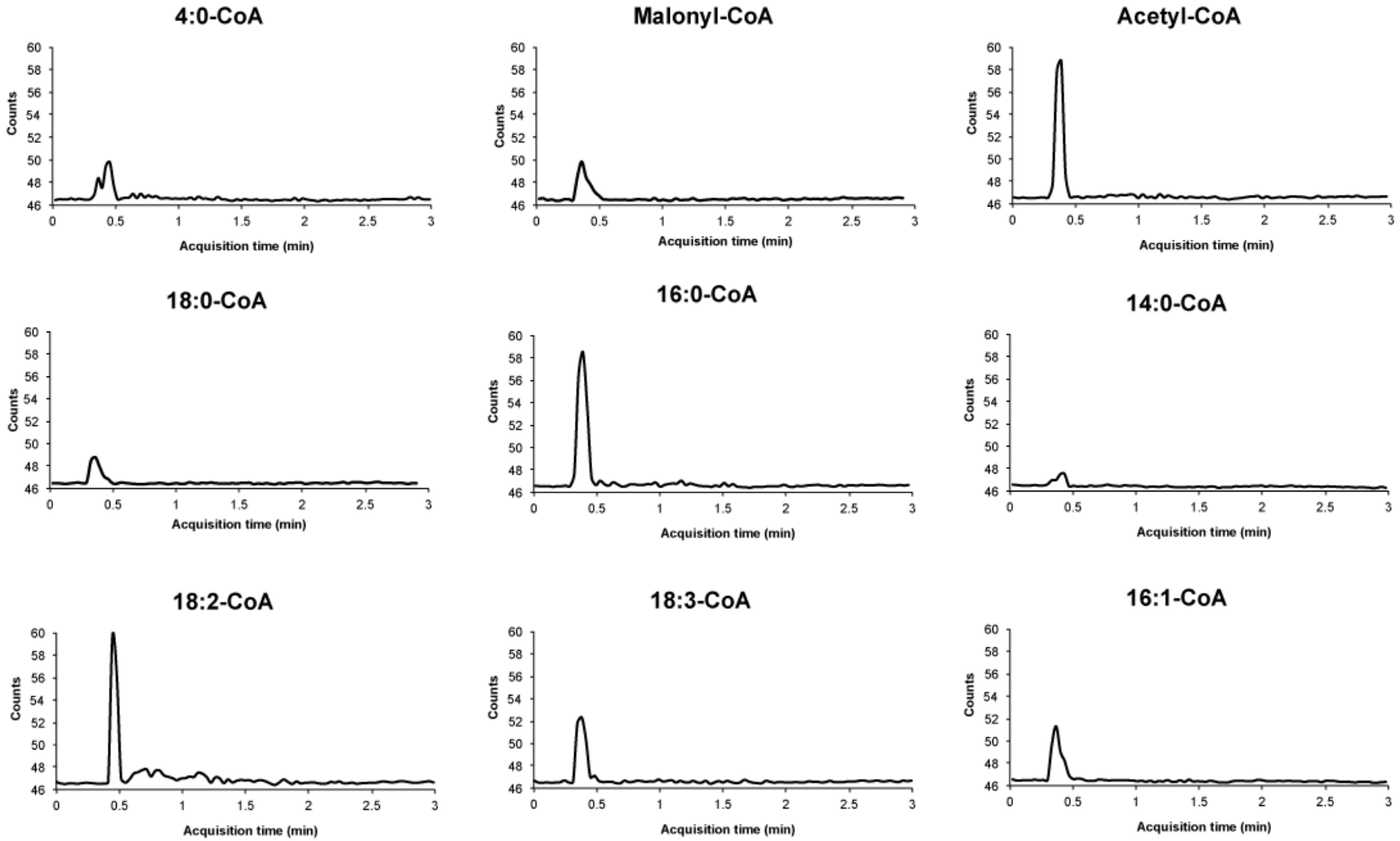


Figure 3.54. Extracted ion chromatograms of each acyl-CoA detected in extracts of the PCC 6803 transformed with *fadD* gene from *E. coli.*

Acyl-CoA synthetase activity can be detected at a significantly higher level in PCC 6803 *fadD* transformants than in wild type PCC 6803. Acyl-CoA synthetase activity was assayed as described in 2.13.3. Activity from acyl-CoA synthetase in crude protein extracts of *fadD* expressing PCC 6803, wild-type PCC 6803 (negative control), *E. coli* (positive control) and no protein extract as a blank was assayed by measuring the incorporation of 1-^{14}C-lauric acid into its coenzyme A thioester, described by the equation:

$$^{14}\text{C-lauric acid} + \text{CoA} + \text{ATP} \rightarrow {}^{14}\text{C-lauroyl-CoA} + \text{AMP} + \text{PPi}$$

The reaction was halted by transferring the mixture to a paper filter disc and washing with an organic solvent; free fatty acid was soluble in the organic solvent, but the CoA thioester was not and remained on the disc. The amount of radioactivity remaining on this disc that was incorporated into lauroyl-CoA was quantified by liquid scintillation counting, with the value of the blank subtracted and expressed as DPM mg protein^{-1} min^{-1}.

A significant increase in average (n = 6) activity was observed in PCC 6803 *fadD* vs. wild-type, increasing by 2.9-fold from 18.5 DPM mg protein^{-1} min^{-1} to 52.5 DPM mg protein^{-1} min^{-1} (Fig 3.55), 2 sample t-test returning p <0.0001 (t = 11.185). *E. coli* crude protein extracts returned an even higher average activity of 163 DPM mg protein^{-1} min^{-1}, this was expected as the enzyme should work more efficiently in its native host compared to PCC 6803. Even though PCC 6803 wt does not have any acyl-CoA synthetase present, a low level of activity could still be detected at 18.5 DPM mg protein^{-1} min^{-1}, this could be due to acyl-ACP synthetase converting the labeled free lauric acid to ACP thioesters that are not soluble in organic solvents either.

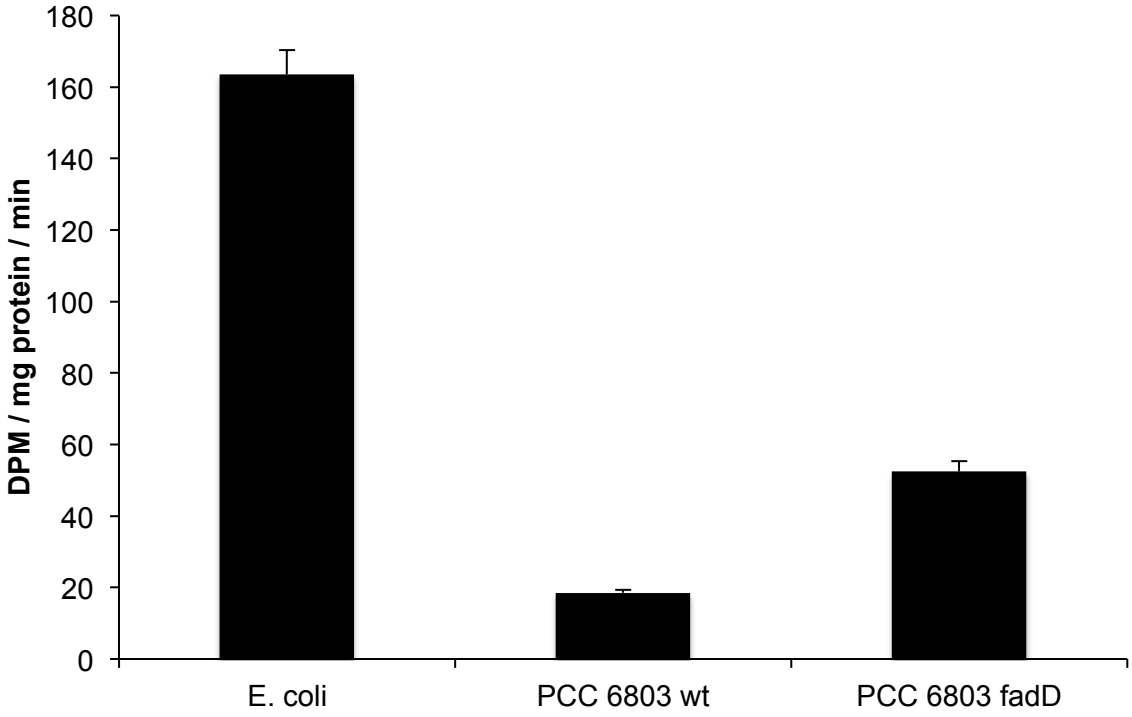


Figure 3.55. Acyl-CoA synthetase activity expressed as DPM of free lauric acid converted to lauroyl-CoA per mg of protein per minute in crude protein extracts of *E. coli*, PCC 6803 wt and PCC 6803 *fadD.* Error bars show SEM (n = 6).

3.2.7 Discussion

Acyl-CoA synthetase from *E. coli* was successfully ectopically expressed in PCC 6803. Expression of *E. coli* acyl-CoA synthetase in PCC 6803 resulted in detection of long-chain acyl-CoAs in cell extracts, where they were previously undetectable in the extracts of the wild-type strain (3.1.3). This only occurred after feeding of free fatty acids. An explanation for this could be that endogenous enzymes that act upon fatty acids, such as acyl-ACP synthetase, are more efficient at scavenging free fatty acids compared to the ectopically expressed acyl-CoA synthetase. An assay to detect and quantify acyl-ACP's, as well as quantification of acyl-CoAs could be used to show whether this is the case.

The BioBrick assembly method and the tools for developed by Huang et al. provided a relatively easy way in which to quickly and efficiently construct an expression system in PCC 6803. Similar methods to the BioBrick method, such as Gateway (Hartley et al. 2000) employ analogous methods of subcloning techniques.

One criticism of the method employed is that only quasi-transient expression can be attained, i.e. in order for the plasmid to remain within the population of cells, it must be selected for by supplementing the cyanobacterial cultures with an antibiotic, such as kanamycin used in this study. Huang et al. reported that the expression plasmid pPMQAK1 was stably maintained in cultures for at least three months, cultures reported here still appear to express *fadD* after six months with antibiotic selection. pPMQAK1 is a broad host range shuttle vector that was developed by Huang et al. directly from pAWG1.1, a plasmid containing the replicon from the IncQ group plasmid RSF1010 (Scholz et al. 1989) that has a broad host range in bacteria (Davison, 2002). This replicon contains the 3 necessary *rep* genes for the plasmids' own replication and maintenance within its host cell (Scherzinger et al, 1984). In addition, three *mob* genes necessary for transfer of the plasmid by conjugation are present, although the plasmid is not self-transmissible, requiring the IncP group RP4-based plasmid pRL443 to

mediate DNA transfer. In order to protect *fadD*-expressing pPMQAK1 from restriction digestion once in its cyanobacterial host, the helper plasmid pRL623, containing AvaI and AvaII methylases, methylates GGNCC sites within the DNA sequence of pPMQAK1, of which there are 4 (Fig 2.2) that these restriction endonucleases cleave (Elhai and Wolk, 1988). Although AvaI and AvaII were originally isolated from *A. varibilis*, isoschizomers of these are found in a wide range of cyanobacteria, including PCC 6803 (Tandeau de Marsac and Houmard, 1987). Growth of pPMQAK1-containing cyanobacteria without antibiotic selection pressure is probably not feasible, as a high copy number is required to maintain replicative stability (Meyer, 2009), copy numbers reported for plasmids derived from RSF1010 in PCC 6803 range from 10 to 30 per cell (Marracini et al. 1993; Ng et al. 2000). In order to attain a fully genetically modified strain, it would be necessary to integrate the desired gene into the chromosome of PCC 6803, this can be achieved by homologous recombination (Okamoto et al. 1999), although another complicating factor is that PCC 6803 contains multiple copies of its chromosome depending upon growth conditions, in one case this was found to be 12 (Labarre et al. 1989), although this is generally more of an issue when attempting to generate gene knock-outs as opposed to ectopic expression.

Further applications of these molecular tools could include moving into the realm of fully synthetic biology, using custom DNA synthesis to introduce the entire operon of fatty acid β-oxidation genes into PCC 6803 by homologous recombination, including the regulatory elements *fadR* and *fabR*. It would be interesting to investigate the pleiotropic effects of having such a pathway introduced into an organism that does not naturally possess one, in particular its effect on the photosynthetic thylakoid membranes and on heptadecane production by the cells, as it is possible that cyanobacteria may produce heptadecane as a safety mechanism to avoid fatty acids reaching toxic levels within the cell, as they are unable to break them down by β-oxidation. Of course, such large changes to the cells' internal biochemistry could also render the cells unviable.

3.3 Acaryochloris strain HICR111A

3.3.1 Introduction

It was mentioned in chapter 3.2 that 2 cyanobacterial strains out of 40 sequenced and annotated on the Kyoto Encyclopedia of Genes and Genomes website have all the genes necessary for β-oxidation present according to electronic annotation, these strains being *Cyanothece* PCC 7425 and *A. marina* MBIC11017. It has been possible to obtain and successfully culture a closely related strain, *Acaryochloris* HICR111A (Mohr et al. 2010). 16S rRNA sequencing conducted by Mohr et al. reveals that this strain groups with PCC 7425 and MBIC11017 (Fig.3.56), so it would be reasonable to assume that this strain also has all β-oxidation genes present.

In order to confirm the presence of an active β-oxidation pathway, experimental evidence was sought by PCR, to determine whether the genes for β-oxidation were present and secondly by RT-PCR to determine if these genes were transcribed. Additionally the same techniques as in chapter 3.2 were employed. It has been shown in the following chapter that HICR111A does not transcribe β-oxidation genes, does not synthesise and accumulate acyl-CoAs; does not show any enzymatic activity from acyl-CoA dehydrogenases or acyl-CoA oxidases and does not incorporate ^{14}C label from 1-^{14}C-decanoic acid into breakdown products resulting from fatty acid degradation.

3.3.2 PCR of β-oxidation genes

16s rRNA control primers were able to amplify from the 16s rRNA genes using the conditions specified by Mohr et al. 2010, showing that gDNA preparations from HICR111A and PCR parameters were satisfactory (Fig. 3.57). PCR products were detectable that matched with estimates of expected PCR products based on sequence homology (Table 2.7) for all genes, except 3-hydroxyacyl-CoA dehydrogenase. At lower annealing temperatures, more nonspecific sequences were amplified. Possible bands on the agarose gel for 3-hydroxyacyl-CoA dehydrogenase were present at annealing temperatures of 53 °C and 54 °C, just above the expected size of 1.4 Kb. These bands were excised from the gel and sequenced, to determine whether these bands had significant homology to other 3-hydroxyacyl-CoA dehydrogenases, but did not have any. To show that this technique works, the 1.2 Kb band amplified from acyl-CoA synthetase was also excised and sequenced, the resulting sequence then translated into protein *in-silico* using 6-frame translation tool; this protein sequence was then analysed using Pfam and identified as being an AMP-binding domain (bit score = 101.0, E-value 4.2e-29), consistent with those found in other acyl-CoA synthetases. It could be possible that HICR111A has a longer version of this gene, as a band at 2.5 Kb was also amplified.

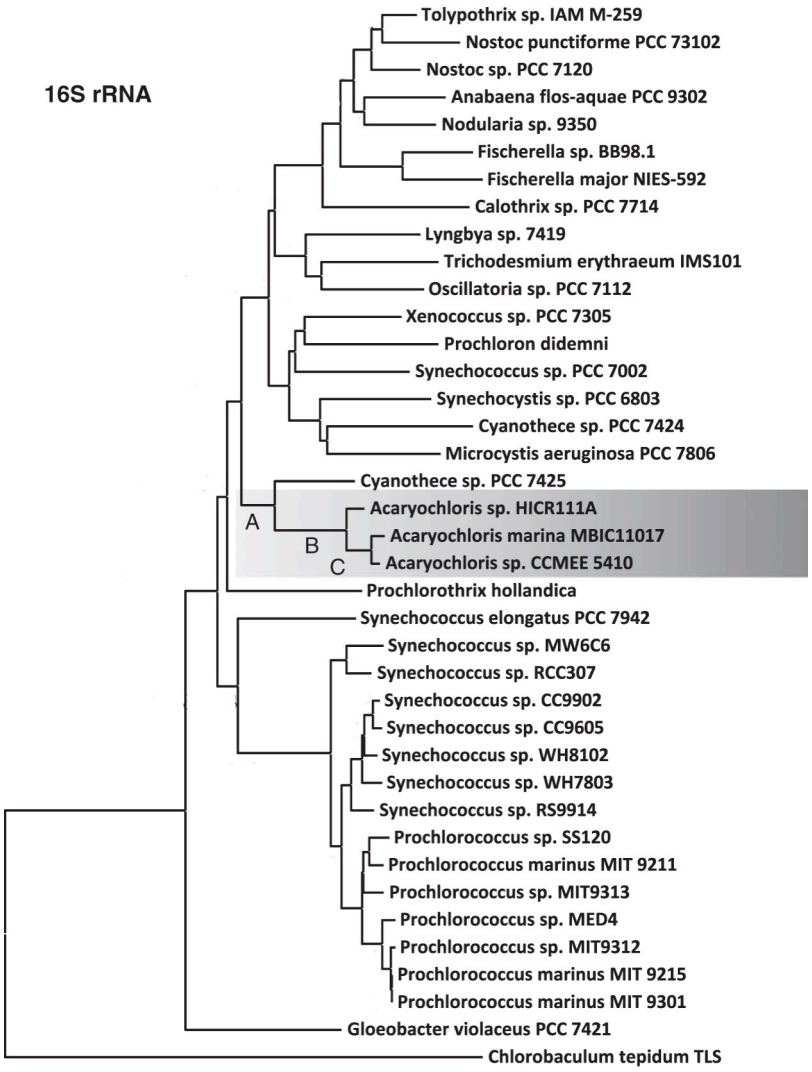


Figure 3.56. Phylogenetic tree based on 16S rRNA sequence comparison. HICR111A groups within node A, along with fully sequenced PCC 7425 and MBIC11017 that have electronically annotated β-oxidation genes. Figure adapted from Mohr et al. 2010.

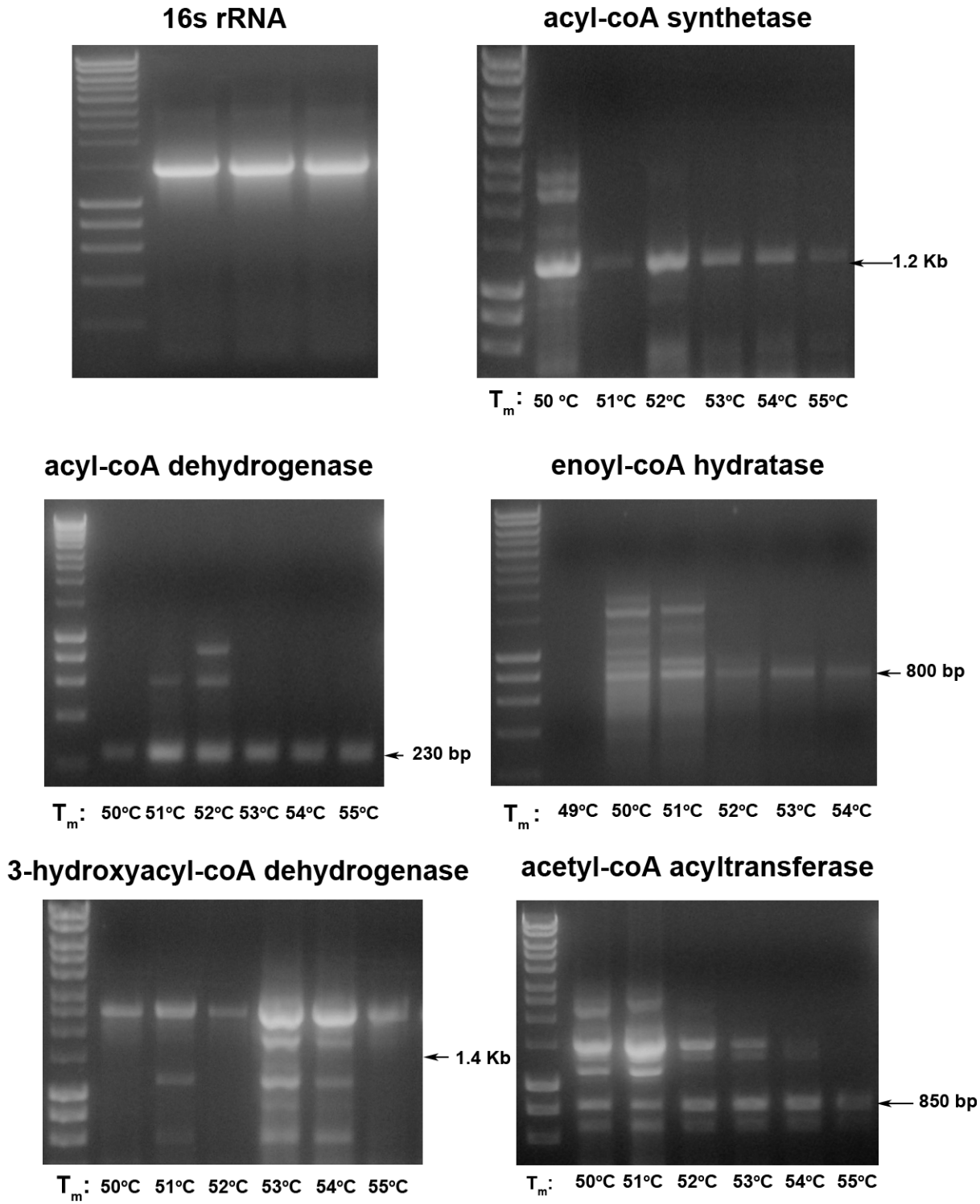


Figure 3.57. Agarose DNA gels of PCR products generated from β-oxidation genes in HICR111A.

3.3.3 RT-PCR of β-oxidation genes

Cultures of HICR111A were grown for 7 days, after which some cultures were moved to a dark incubator with no supplemented CO_2 for 2 days, before being fed with 0.3 mM decanoic acid, in order to try to 'induce' expression of β-oxidation genes. Cultures were harvested and RNA was extracted, yield being in the region of 6.3 μg from 25 ml of culture. RNA was converted to cDNA by reverse transcriptase and PCR was performed with the same primers and conditions used to amplify gDNA (see 2.16.6 and 3.3.2). The 16S rRNA sequence was successfully amplified, however none of the correct PCR product sizes could be detected for any of the β-oxidation transcripts (Table 2.7 and Fig. 3.58). A PCR product could be detected for enoyl-CoA hydratase transcripts, but this product was twice the length of the expected band, being 1.6 Kb versus 0.8 Kb. In order to determine whether this was a dimer or extended version of the expected sequence, the PCR product could have been cut out and sequenced. However as none of the other sequences were present, there was little point in doing this, as the four preceding enzymes in this pathway are essential to β-oxidation. There was no difference between the 'induced' and non-induced cDNA preparations' results.

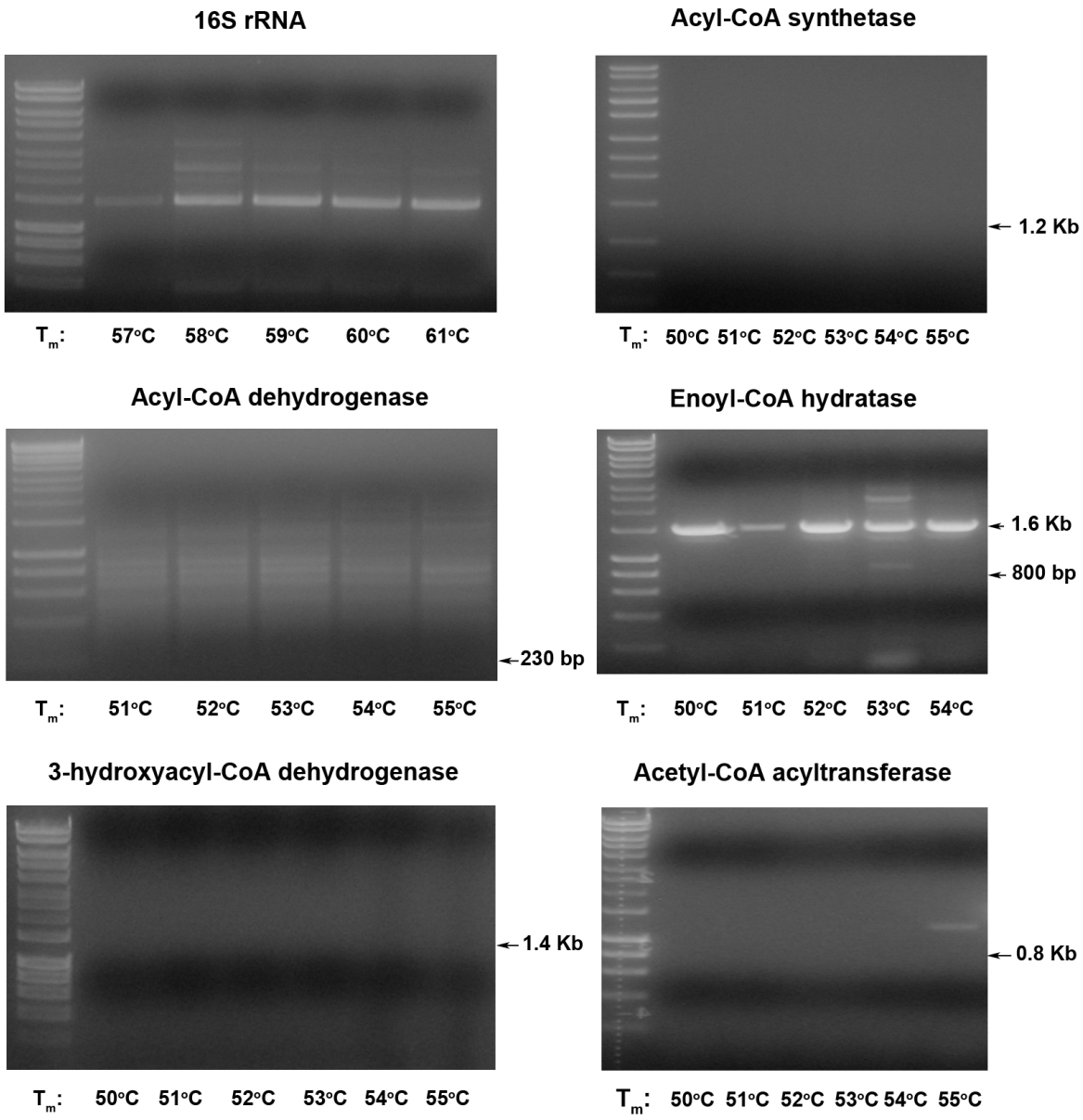


Figure 3.58. Agarose DNA gels of PCR products generated from β-oxidation cDNA transcripts in HICR111A.

3.3.4 Acyl-CoAs LCMS Assay

Acyl-CoAs were extracted, enriched and detected by triple quadrupole LC-MS as described in 3.1.3. The only acyl-CoAs detectable in HICR111A were acetyl-CoA and malonyl-CoA (Fig. 3.59). In order to show that other components of the cyanobacterial extracts were not inhibiting ionisation or detection of acyl-CoAs, 150 pmol 18:0-CoA was spiked into the extract and sample was re-injected (Fig. 3.60).

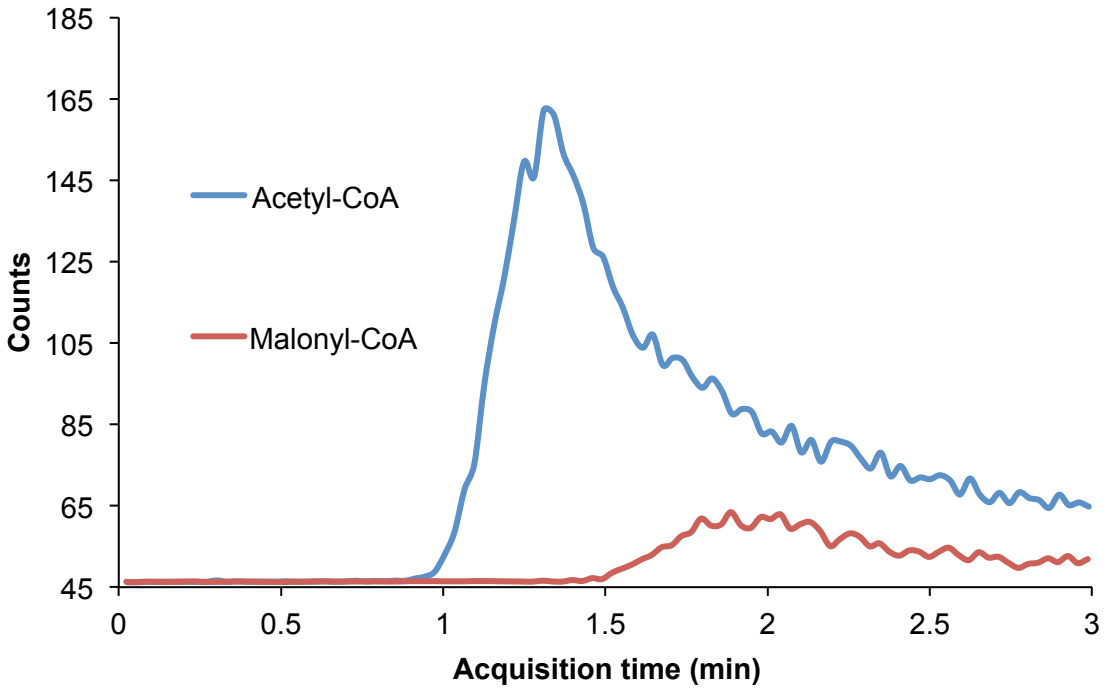


Figure 3.59. Extracted MRM chromatograms for acetyl-CoA and malonyl-CoA in extracts of HICR111A.

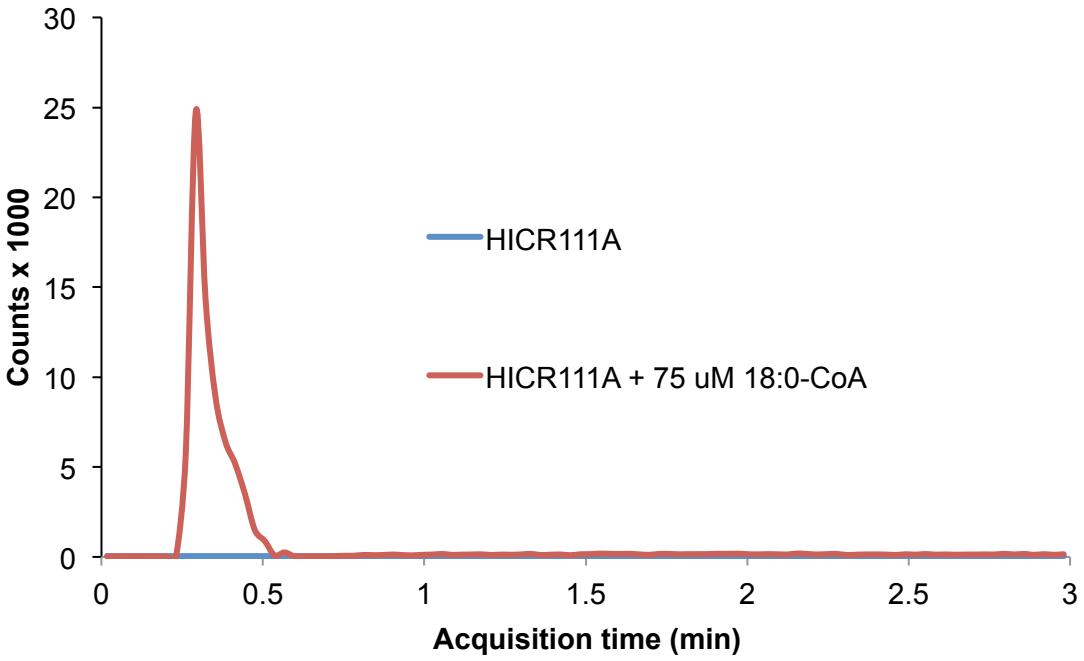


Figure 3.60. Extracted MRMs for 18:0-CoA from extracts of HICR111A alone and with 150 pmol spiked into the same extract.

3.3.5 No activity for acyl-CoA dehydrogenase or acyl-CoA oxidase can be detected in strain HICR111A

Acyl-CoA dehydrogenase and acyl-CoA oxidase were assayed as described in 2.13 and 3.2.4. As in strains PCC 6803, PCC 7120 and PCC 7937, negligible activity could be found above baseline rate of reduction of INT for acyl-CoA oxidase (Fig. 3.61) or baseline rate of purpurogallin produced for acyl-CoA oxidase (Fig. 3.62).

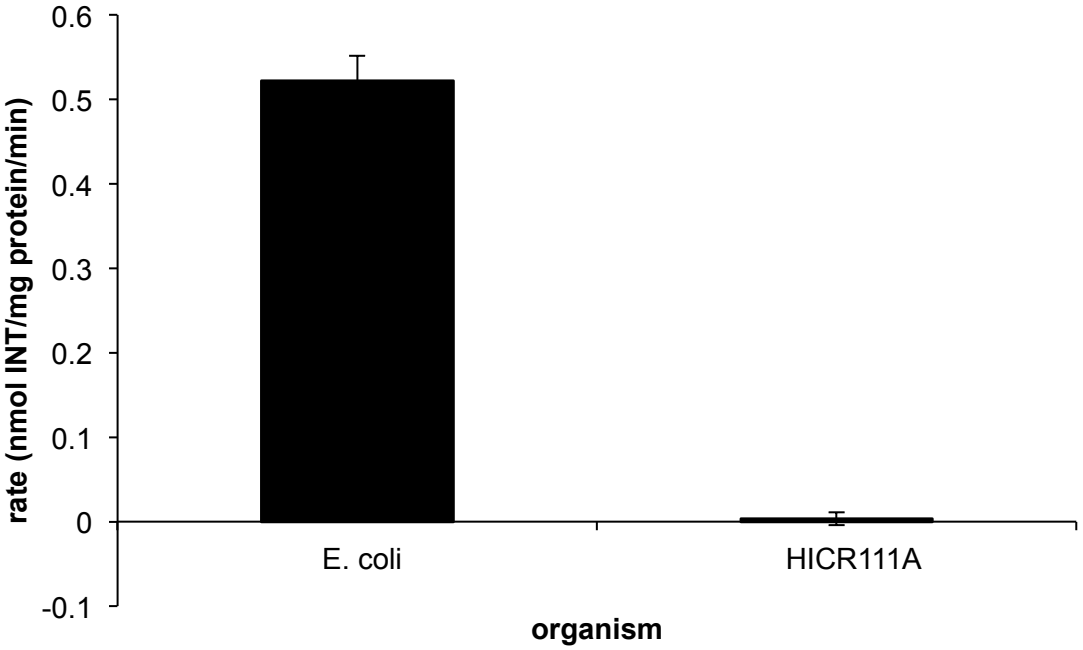


Figure 3.61. Comparison of initial rates of acyl-CoA dehydrogenase activity as quantified by nmol of INT reduced per mg of protein in crude extract per minute in *E. coli* and HICR111A. Error bars show S.E. n = 6.

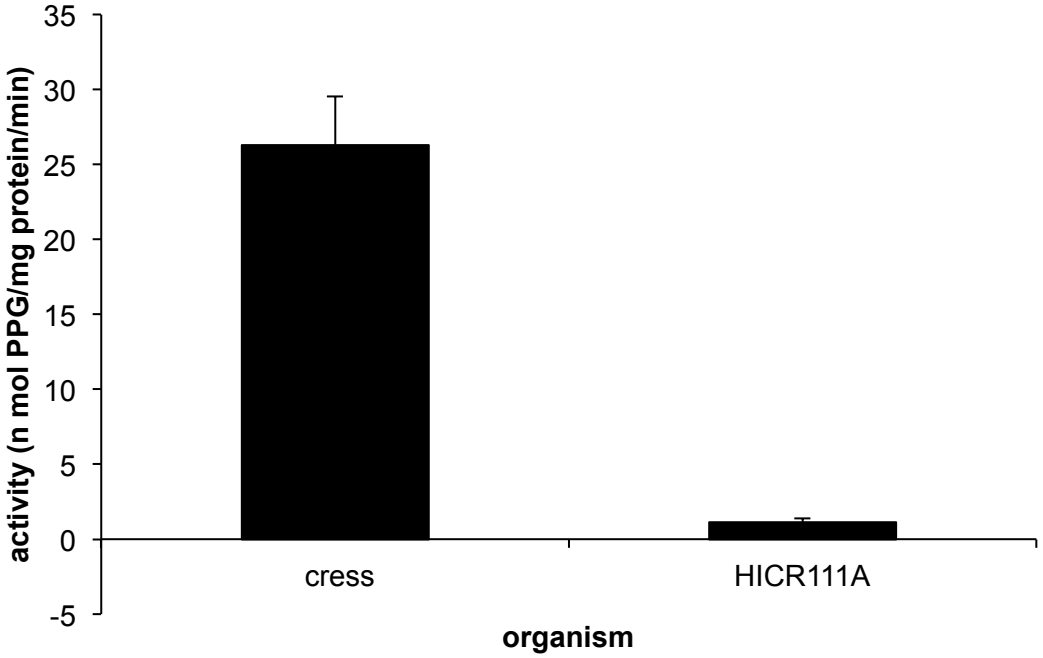


Figure 3.62. Comparison of initial rates of acyl-CoA oxidase activity as quantified by nmol of purpurogallin produced per mg of protein in crude extract per minute in cress, PCC 6803, PCC 7120 and PCC 7937. Error bars show SEM n = 3.

3.3.6 Metabolism of ^{14}C-labelled fatty acids

HICR111A was fed 1-^{14}C-decanoic acid for 1 h in the dark, resuspended in label-free medium, and samples taken at 1 h, 4 h, 8 h and 20 h intervals as described in 3.1.5. Samples were extracted as described in methods section 2.9 and subject to analysis by scintillation counting, TLC and LC-QToF-MS in order to look for resulting products arising from fatty acid biosynthesis or metabolism.

HICR111A was observed to take up on average (n=3) 90% of 1-^{14}C-decanoic acid from the media after 1 hour (Fig. 3.63, E), higher than observed in *E. coli* and comparable to PCC 6803 (Fig. 3.28, F), however after this point label was seen to increase in media from a low of 1.7 % after 4h up to 2.7 % after 8 h, indicating that a small amount of label was being re-introduced back into the medium, as in *E. coli,* albeit not to as high a degree. After 20 h total activity in the media has dropped back to 2.2 %. No radioactivity could be detected at any timepoint in the media by TLC.

$^{14}CO_2$ was released from 1-^{14}C-decanoic acid at a similar rate to PCC 6803 (Fig. 3.63, A and B), 1.1 % of total radioactivity from 1-^{14}C-decanoic acid had been released after 20 h, compared to 1.3 % in PCC 6803. These values are 17-fold lower than observed in *E. coli*.

60 % of total ^{14}C label from 1-^{14}C-decanoic acid was found to be in the aqueous phase, this dropping down to a low of 20 % after 8 h and stayed at that level for the remainder of the timecourse (Fig. 3.63, C). This response was not observed in other cyanobacteria and the drop in activity in this phase is even more extreme than was observed in *E. coli* (Fig. 3.36, C), but unlike in *E. coli* there was a concomitant rise in label incorporated into lipids (Fig. 3.63, D) at 4 h, rising to 60 % from 20 % at 1 h, the opposite of what was observed in the aqueous phase. After 4 h, activity remained at 60 % for the rest of the timecourse. Analysis of the aqueous phase using TLC showed that radioactivity present at 1 h moved with 2 spots, one at the solvent front and the other just below (R_f = 0.8) that were not present at the later timepoints (fig, 3.64, A), unlike in

other strains of cyanobacteria, where the spots of radioctivity resulting from fatty acids were present throughout the timecourse (figs. 3.38, B; 3.39, B and 3.40, B). Analysis of these upper spots using LC-QToF-MS showed that these were composed mainly of 16:0 and 18:0 fatty acids (M-H = 256.2401 and 284.2713 m/z), with little 10:0 (M-H = 171.2523 m/z) present (Fig. 3.65). Other fatty acids were also present at lower abundances (Fig. 3.66). No amino acids, organic acids or sugars incorporating label could be detected by TLC.

Analysis of the DCM phase by TLC showed that a large percentage of label from 1-^{14}C-decanoic acid was incorporated into complex lipids (Fig. 3.64, B and C), as in other cyanobacteria (figs. 3.30, C; 3.31, C and 3.32, C). Fatty acids can clearly be seen to disappear after 1 h on the petroleum ether/DCM/acetic acid solvent system, backing up the observations of activity disappearing also in the aqueous phase.

Analysis of regions where label was observed to migrate to in the acetone/toluene/water solvent system in non-radiolabeled decanoic acid fed cultures by LC-QToF-MS show that only fatty acids and no complex lipids were detected at the solvent front, the composition of fatty acids did change over the timecourse, with more stearic acid present at 20 h compared to the start (Table 3.17), however the amount of decanoic acid did not decrease, actually a slight increase in decanoic acid was observed, from 5.8 % total fatty acids at 1 h to 6.7 % total fatty acids at 20 h.

The upper spot was found to contain 6 MGDGs (Table 3.18), interestingly one of these being a 10:0 acyl chain. The lower spot contained the glycolipids DGDG, SQDG and the phospholipid PG(16:0/18:2).

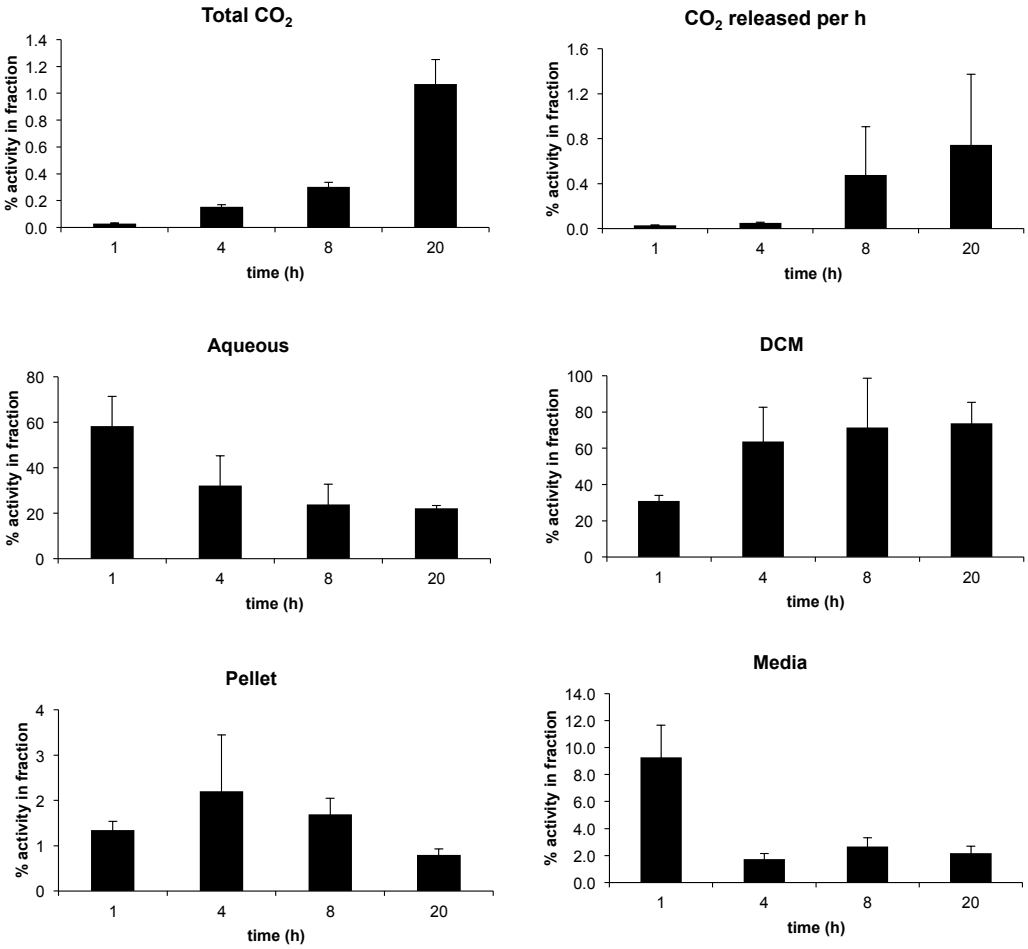
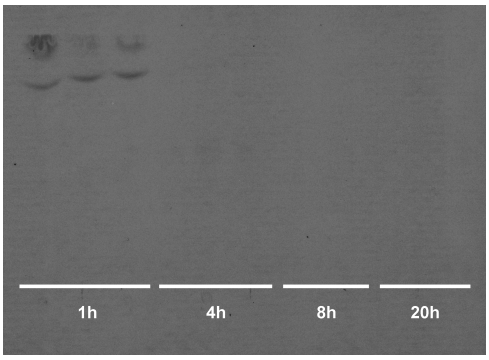
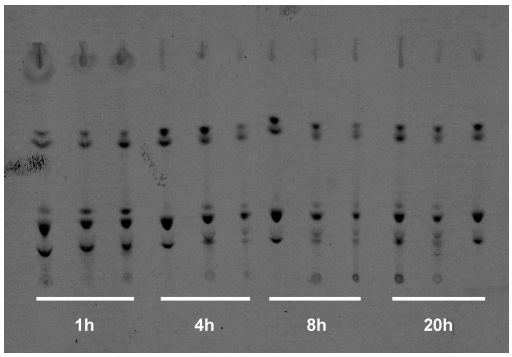


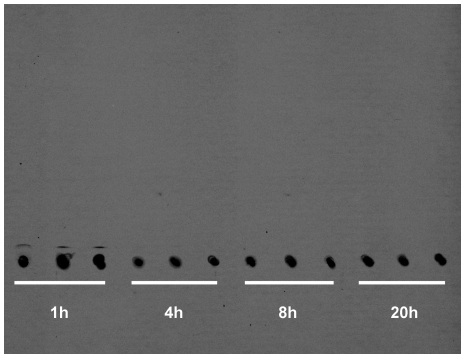
Figure 3.63. Percentage of total ^{14}C label from 1-^{14}C-decanoic acid incorporated into different fractions by HICR111A over a 20 h period. Error bars show S.E. n = 3



A: Aqueous fraction



B: Complex lipid fraction



C: Hydrocarbon fraction

Figure 3.64**.** Autoradiograms of TLCs of aqueous and DCM fractions in PCC 6803. Aqueous fraction (A) was separated with ethanol/18.1 M ammonia 7:3 v/v solvent system; “complex lipids” (B) separated with acetone/toluene/water 90:30:8 v/v/v solvent system; “hydrocarbons” (C) separated with petroleum ether/DCM/acetic acid 90:10:1 v/v/v.

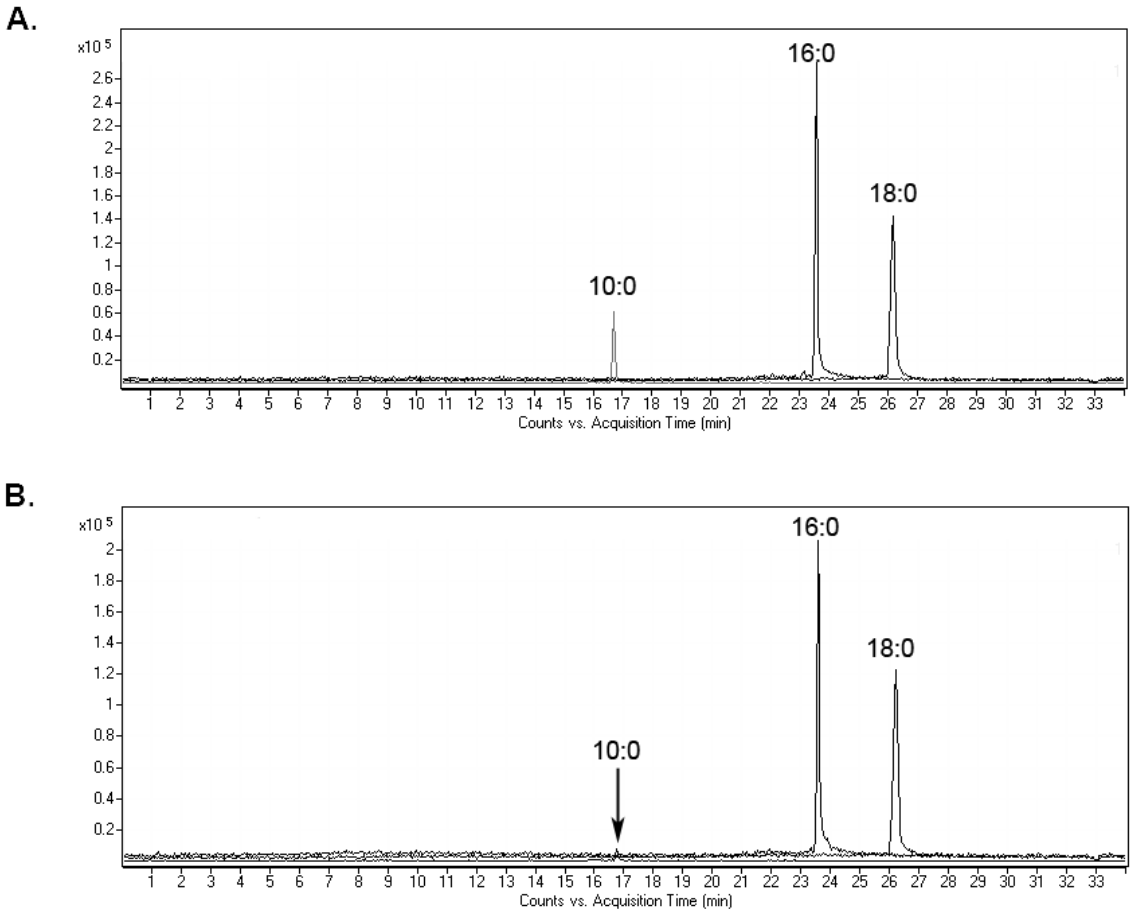


Figure 3.65. EIC for decanoic acid (10:0; 171.2523 m/z) palmitic acid (16:0; 256.2401 m/z) and stearic acid (18:0; 284.2713 m/z) from single QToF runs of spots where radioactivity from ^{14}C was found to be on TLC plates of the aqueous phase in HICR111A. **A**. Upper spot. **B.** Lower spot.

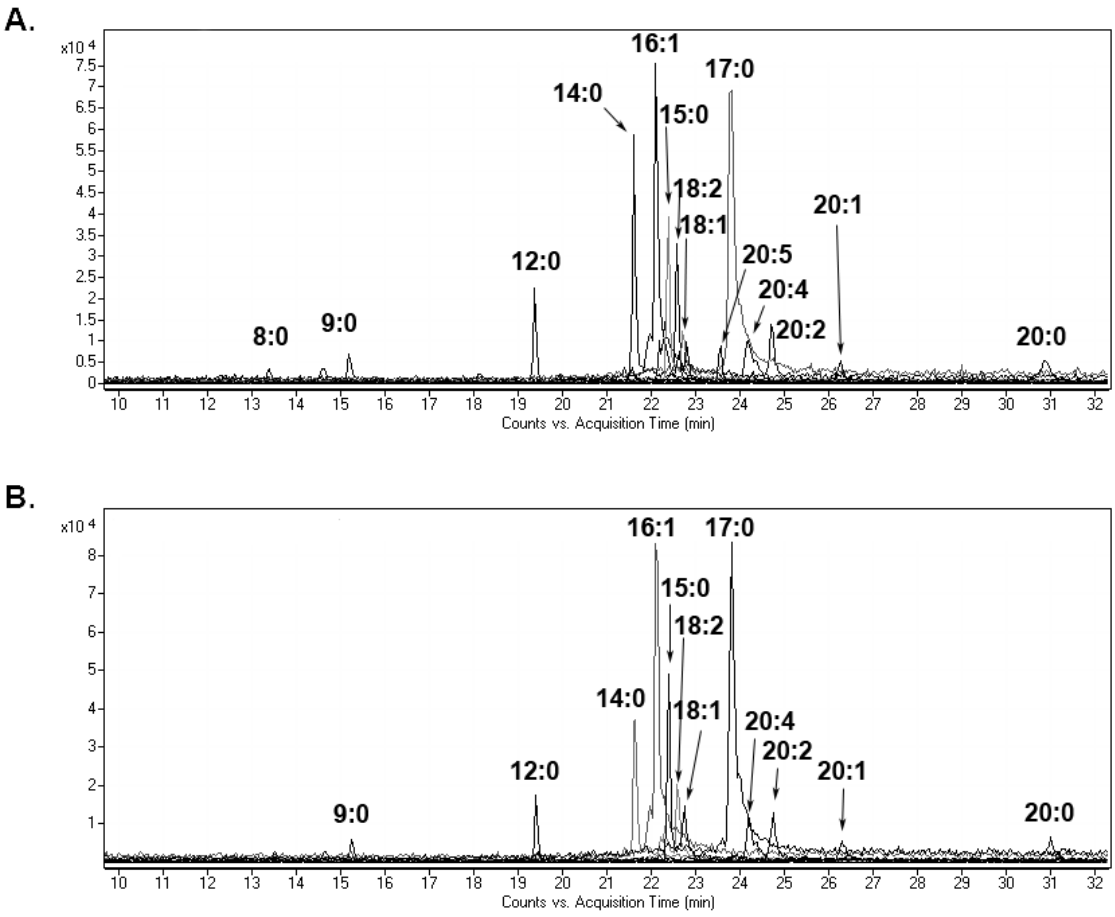


Figure 3.66. Extracted ion chromatograms of other fatty acids detected at lower abundance than in Fig. 3.65. Data was obtained from single QToF runs of spots where radioactivity from ^{14}C was found to be on TLC plates of the aqueous phase in HICR111A. **A.** Upper spot. **B.** Lower spot.

Table 3.17. Fatty acid composition of spot at solvent front in acetone/toluene/water solvent system 1 h and 20 h after feeding of HICR111A.

Fatty acid	**% of total fatty acids**	
	1 h	**20 h**
10:0	5.8	6.7
12:0	0.3	1.9
14:0	2.2	5.1
15:0	0.7	1.5
16:0	71.1	50.6
16:1	0.5	0.4
17:0	4.9	0.7
18:0	5.8	24.1
18:1	6.6	3.4
18:2	0.1	0.4
18:3	ND	1.5
18:4	ND	0.4
20:0	0.4	2.0
20:3	0.4	1.1
20:5	1.2	ND

Table 3.18. Profile of complex lipids in HICR111A that migrated on TLC plate where activity was detected from 1-^{14}C-decanoic acid feeding.

Spot	**Compound**	**Precursor ion m/z**	**Mass accuracy Δppm**	**Notes**
Upper	MGDG(16:0/18:2)	745.4981	37	
	MGDG(16:0/18:3)	797.5377	3	
	MGDG(16:0/18:4)	683.3279	2	
	MGDG(16:0/10:0)	691.4243	23	
	MGDG(16:0/12:0)	733.5029	52	
	MGDG(16:0/12:1)	717.4411	53	
Lower	SQDG(21:1)	711.3584	1	Not possible to determine acyl chain composition from MS/MS data
	SQDG(22:1)	725.3782	6	
	SQDG(33:4)	815.4935	5	
	SQDG(33:3))	817.5113	5	
	DGDG(16:0/0:0)	811.4325	1	
	DGDG(16:0/10:0)	853.4818	39	
	DGDG(16:0/12:0)	881.5479	41	
	DGDG(16:0/13:1)	893.5479	42	
	PG(16:0/18:2)	745.4975	1.7	

3.3.7 Discussion

From the data acquired in this chapter, it appears that HICR111A cannot degrade fatty acids, like other cyanobacteria. All genes required for β-oxidation, except 3-hydroxyacyl-CoA dehydrogenase, appear to be present according to PCR. This shows that these sequences bear significant homology to those found in MBIC11017 and PCC 7425. It is possible that 3-hydroxyacyl-CoA dehydrogenase is not present in HICR111A or that is does not bear a close enough homology to its orthologs found in MBIC11017 and PCC 7425, whose genomic DNA sequences were used as templates for primer design. Another possibility is that the sequence for this particular enzyme is longer that what the extension time allowed for in the PCR.

In order to determine whether these genomic β-oxidation sequences were transcribed into mRNA, a qualitative-only RT-PCR approach was used. As 16S rRNA was able to be amplified, but mRNA transcripts were not, there is strong evidence to support the argument that the β-oxidation sequences found in HICR111A could be pseudogenes. The type of reverse transcriptase used in this kit utilized random 6-nucleotide primers as well as polyA primers that are commonly used to reverse transcribe eukaryotic mRNA sequences, hence how rRNAs were also able to be converted to cDNA; prokaryotic mRNAs do not usually contain polyA tails, as they promote degradation of mRNAs (Steege, 2000). As these β-oxidation sequences could be pseudogenes in HICR111A, it could also be supposed that other members of the *Acaryochloris* genus, as well as PCC 7425 all possess β-oxidation pseudogenes, as they are very closely related organisms according to 16S rRNA sequence homology (Fig. 3.56).

Further experimental evidence supporting the above conclusions are shown by the lack of long chain acyl-CoAs present in extracts, lack of enzymatic activity arising from acyl-CoA dehydrogenases or oxidases and a lack of breakdown products observed when cultures are fed radiolabeled fatty acid. However HICR111A did show metabolism of ^{14}C decanoic acid slightly different to that of the other 3 cyanobacteria strains tested,

with no activity at all detected in the medium after 1 h and activity in the aqueous phase disappearing after 1 h as opposed to it remaining there throughout the timecourse as in other cyanobacterial strains; although an explanation for this could be due to the fatty acids being incorporated into complex lipids. Lipid profiles of HICR111A were similar to that of other cyanobacteria, showing similar TLCs and complex lipids, although only 1 17:0 fatty acid peak could be observed in HICR111A, opposed to 2 peaks in other cyanobacteria (compare Figs. 3.66 and 3.34), meaning that HICR111A does not make either one of the branched or straight chain 17:0 fatty acids, unlike the other cyanobacteria that have both isomers present. If the β-oxidation sequences in the cyanobacteria are all pseudogenes, then it could mean that β-oxidation is a very ancient metabolic pathway that the cyanobacteria have seemingly lost the ability to use or the pathway has become dispensable due to the photoautotrophic lifestyle that cyanobacteria lead, although plants and higher algae largely have intact β-oxidation pathways. This could also provide evidence that *Acaryochloris* is one of the more ancient lineages of cyanobacteria, as β-oxidation pseudogenes are present. Strain PCC 7425 could perhaps be reclassified as member from its current genus of *Cyanothece*, as it appears to have more homology with *Acaryochloris* members according to both 16S and β-oxidation sequences, although a defining feature of the *Acaryochloris* genus is having the presence of chlorophyll d that utlises far-red and near infrared wavelengths of light (Miyashita et al. 1996) that PCC 7425 is not known to do. *Acaryochloris* are thought to live in deep marine environments, where near infrared light is abundant (Kuhl et al. 2005) compared to other wavelengths that have been filtered out by depth of the water, compare this to PCC 7425 that was isolated from a freshwater rice field in Senegal (Rippka et al. 1979), which have no need to utilize far red wavelength light. That PCC 7425 and *Acaryochloris* strains have such a high level of sequence conservation yet live in such diverse environments gives further evidence to the ancient lineage of the cyanobacteria.

3.4 Regulation of fatty acid biosynthesis in *Synechocystis* PCC 6803

3.4.1 Introduction

As strains PCC 6803, PCC 7120, PCC 7937 and HICR111A have been shown to lack an active β-oxidation pathway, it is reasonable to suppose that cyanobacteria may have evolved a highly regulated fatty acid biosynthetic pathway to ensure that minimal energy is wasted, because if too much fatty acid is synthesized then this cannot be re-oxidised for the production of energy or other structural compounds. As described in 1.8, other bacteria are known to regulate fatty acid biosynthesis and degradation primarily at the transcriptional level, but also by feedback inhibition. In *E. coli* this is performed by the transcriptional repressor FabR (Fujita et al. 2007), the transcription repressor/activator FadR (Henry and Cronan, 1992) and also by direct feedback inhibition of FabH (KASIII) , FabI (enoyl-ACP reductase) and the acetyl-CoA carboxylase complex enzymes by acyl-ACPs (Heath and Rock, 1996). Metabolism of fatty acids in other bacteria that have been characterized in the literature include *Bacillus subtilis* and *Streptococcus pneumoniae,* that utilize orthologous systems of transcriptional repression (Matsuoka et al. 2007; Lu and Rock, 2006), with some differences, for example the FabR ortholog, FapR in *B. subtilis,* is known to be inhibited by feedback from malonyl-CoA, whereas the signal that antagonizes binding of *E. coli* FabR is still unclear. Chloroplasts of higher plants have been shown to regulate fatty acid biosynthesis by direct feedback inhibition of the acetyl-CoA carboxylase alone by acyl-ACPs, with no regulation observed at the transcriptional or translational level (Andre et al. 2012).

Sequences of the transcriptional regulators described above show low homology when aligned against published cyanobacterial genomes (Table 3.19), compared to an ortholog that are found in both *E. coli* and cyanobacteria (an RNA polymerase subunit from *E. coli*), which suggest that such regulators are either not present as is proposed in plants, or are significantly divergent from those found in the 3 bacterial species

described above. Interestingly, 3 of the best matches out of the 5 regulator sequences searched against the 40 cyanobacterial genomes were those from MBIC11017 and PCC 7425, the two strains that have β-oxidation genes present according to computational annotation (Table 3.1), although these scores are low compared to *E. coli* RpoC.

Table 3.19. Best scoring BLAST hits of 40 cyanobacterial strains against the protein sequences of 5 fatty acid metabolism transcriptional regulators from *E. coli*, *B. subtilis* and *S. pneumoniae,* showing low scores in comparison to a known ortholog – *E. coli* RpoC RNA polymerase.

Regulator	**Best score**	**E-value**	**In organism**	**Ontology**
E. coli FabR	49.7	9e-07	*A. marina* MBIC11017	TetR family transcriptional regulator
E. coli FadR	42.4	6e-04	*Cyanothece* PCC 7425	GntR family transcriptional regulator
B. subtilis FapR	31.6	0.33	*A. variabilis* PCC 7937	Hypothetical GntR family transcriptional regulator
B. subtilis FadR	62.4	1e-11	*A. marina* MBIC11017	Transcriptional regulator
S. pneumoniae FabT	55.5	7e-10	*Cyanothece* PCC 7822	MarR transcriptional regulator
E. coli RpoC	654	<1e-99	*G. violaceus* PCC 7421	DNA-directed RNA polymerase subunit gamma

In order to determine if expression of fatty acid biosynthesis genes was affected by regulatory elements, semi-quantitative RT-PCR was used to assess their level of expression. Expression at differing stages of growth and under the presence of excess fatty acid were assessed. A second experiment assessing the efficiency of incorporation of acetate into fatty acids under conditions of excess fatty acid, using ^{14}C labeling was conducted to investigate possible feedback inhibition.

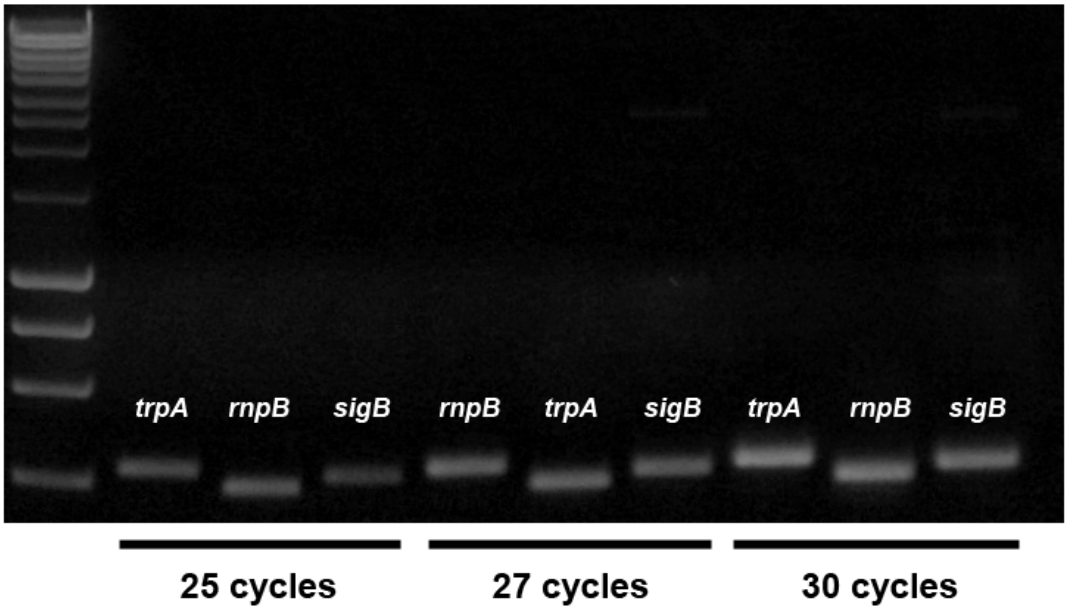
3.4.2 Investigating regulation of fatty acid biosynthesis at the transcriptional level

Semi-quantitative RT-PCR was used to investigate the regulation of fatty acid biosynthesis at the transcriptional level, by comparing the expression level of fatty acid biosynthesis genes under various conditions. This was achieved by extracting RNA from PCC 6803 cultures, converting 1 μg of RNA to complementary DNA (cDNA) using reverse transcriptase and performing PCR. Primers were designed to amplify a 400 bp fragment of the cDNA transcript. Fragments were separated on an agarose gel, the gel was photographed and DNA band intensity analysed in ImageJ.

Three control genes were employed that have been shown to maintain a consistent expression pattern. *trpA* and *rnpB* are genes encoding the tryptophan synthetase A subunit and the ribonuclease B subunit respectively and are unaffected by chemical treatment and different light/dark conditions, having been used to investigate nitrogen metabolism in PCC 6803 (Alfonso et al. 2001). *sigB*, also termed *rpoD,* has been shown to retain consistent expression throughout the course of a transcriptomics experiment conducted by another lab member (unpublished data, Eleanor Jameson, University of Exeter 2009).

In order to normalize the expression levels of fatty acid biosynthesis genes throughout experiments, *trpA* was selected as the control gene as it had the most consistent level of intensity (Table 3.20) with the lowest standard deviation in band intensity. The number of cycles of the PCR was varied to determine the region where amplification of transcripts was linear, the linear region was determined to be in the region of 27 cycles (Fig. 3.67).

A.



B.

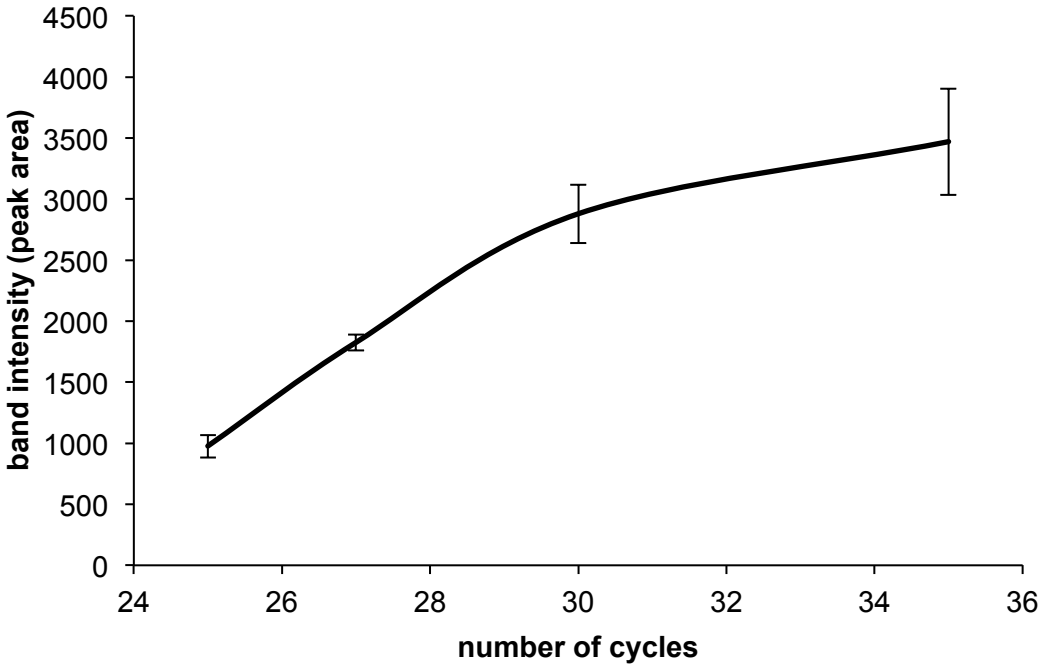


Figure 3.67. Optimization of PCR in order to determine the number of cycles where amplification remains linear. **A.** DNA gel showing increasing PCR product brightness for each of the control genes *trpA, rnpB* and *sigB* for 25, 27 and 30 cycles respectively. **B.** Plot of average band intensities of 3 control genes against cycle number. Error bars show SEM n = 3.

Table 3.20. Comparison of control gene average band intensities as quantified by ImageJ over 26 experiments, plus respective standard deviations.

Gene	**Average intensity**	**S.D**
trpA	4361.8	443.82
rnpB	5677.5	580.47
sigB	6300.1	766.99

Initially the effect of growth phase on the profile of fatty acid biosynthesis genes was investigated, as it is reasonable to assume that more biosynthesis of structural molecules, i.e. fatty acids and lipids, would be occurring in young cultures that are in the phase of exponential growth, compared to cultures that are in stationary phase. However no significant differences in expression profiles of fatty acid biosynthesis genes could be detected between these two growth phases (Fig. 3.68). Cultures in exponential phase were harvested four days after subculture and cultures in stationary phase were harvested ten days after subculture. Perhaps if the cultures were harvested at 20 days, well into late stationary phase, then a larger difference would have been observed.

The next experiment focused on feeding cyanobacteria a range of fatty acids, as well as acetate and ethanol, to investigate whether these had an effect on the expression of fatty acid biosynthesis at the transcriptional level. Acetate is a precursor to fatty acids, as acetyl-ACP, and so could be expected to have a positive effect on the expression of fatty acid biosynthesis genes. The expression level of fatty acid biosynthesis genes in PCC 6803 when fed acetate was compared with no feeding. Three fatty acids - decanoic acid, a medium chain fatty acid, and palmitic and stearic acid, two long chain fatty acids, were fed to PCC 6803, however due to their low solubility in water, these

compounds were dissolved in 100% ethanol and spiked in at a working concentration of 1 % v/v (see 2.16.7 and 6.2). As a control for these three fatty acids, ethanol was fed to PCC 6803. Refer to Fig. 1.4 for an overview of fatty acid biosynthesis, and the names of respective genes described below.

Significant decreases were observed in the expression of *accB*, *accC* and *accD* in acetate-fed cultures of PCC 6803 (Fig. 3.69), having 3.5-fold, 1.9-fold and 1.2-fold lower levels of relative expression compared to the unfed control sample; ANOVA statistical tests having *p* values <0.0001 for *accB, accC*, and *accD* (F ratios of 143.603, 289.171 and 112.502 respectively, well above the critical value of 5.79). No significant changes in expression were observed in any of the *fab* genes or the *cer8* homolog, the gene encoding acyl-ACP synthetase involved in fatty acid recycling (*slr1609*), also termed *aas*. Decanoic acid did not have an effect on the expression of any of the *acc* genes, but did cause a 3.1-fold inhibition of *fabI* compared to the ethanol control, ANOVA returning a *p*-value and F ratio of 0.004 and 6.455. Palmitic acid fed cultures displayed a large degree of inhibition of *accB, accC* and *accD*, having 2.5-fold and 2.1-fold lower expression level than the ethanol control for *accB* and *accD*, and 15.6-fold lower expression than the ethanol control for *accC*, *p* <0.0001 for *accB* and *accC*, and 0.002 for *accD*). In addition, a significant upregulation in *cer8* was observed in cultures that were fed palmitic acid versus the ethanol control, showing a 1.8 fold higher level of expression compared to the ethanol control (*p* = 0.029, F = 6.455). No significant change in the level of expression of the fatty acid biosynthesis genes tested were observed in stearic acid fed cultures compared to the ethanol control – this could be due to the low solubility of this compound in ethanol, so it was not in an available state for the cyanobacteria to utilize. Another observation is that spiking 1 % v/v ethanol into the mixture generally caused a reduction in the relative expression of the *acc* genes compared to the unfed samples, perhaps due to stress, or possibly because the ethanol was converted to acetate in the cyanobacteria by enzymes with ethanol dehydrogenase and acetaldehyde dehydrogenase activity. The expression of *fab*

genes in ethanol fed cultures appeared to have a high degree of variability, hence the large error bars in some samples; this presented some difficulties for the reliable quantification of expression of *fab* transcripts in cultures that were fed fatty acids as they were dissolved in ethanol. The apparent inhibition of fatty acid biosynthesis caused by acetate could be caused by a downstream metabolite, such as acyl-ACPs or fatty acids resulting from increased fatty acid biosynthesis or malonyl-CoA, which is known to repress fatty acid biosynthesis in *B. subtilis* and the concentration of which is increased in PCC 6803 when fed acetate (Fig. 3.71), as determined by the LC-MS method to detect acyl-CoAs as described in 3.2.3.

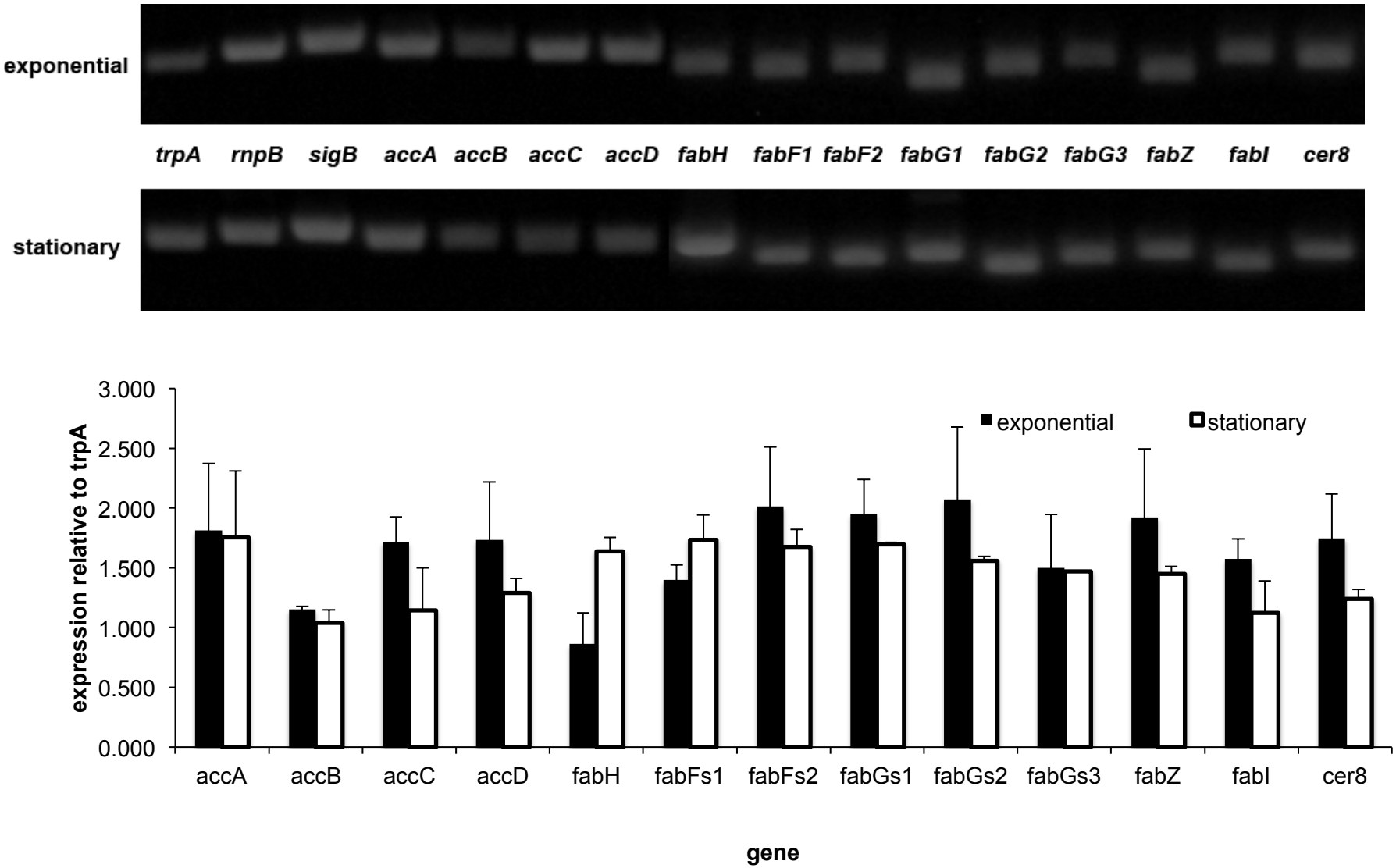


Figure 3.68. Effect of growth phase on expression of fatty acid biosynthesis genes in PCC 6803. **A.** DNA gel images of amplified PCR products of respective transcripts from PCC 6803 cDNA. **B.** Expression of fatty acid biosynthesis gene s relative to control gene *trpA* Black bars indicate exponential phase and white bars indicate stationary phase. Error bars show SEM n = 4.

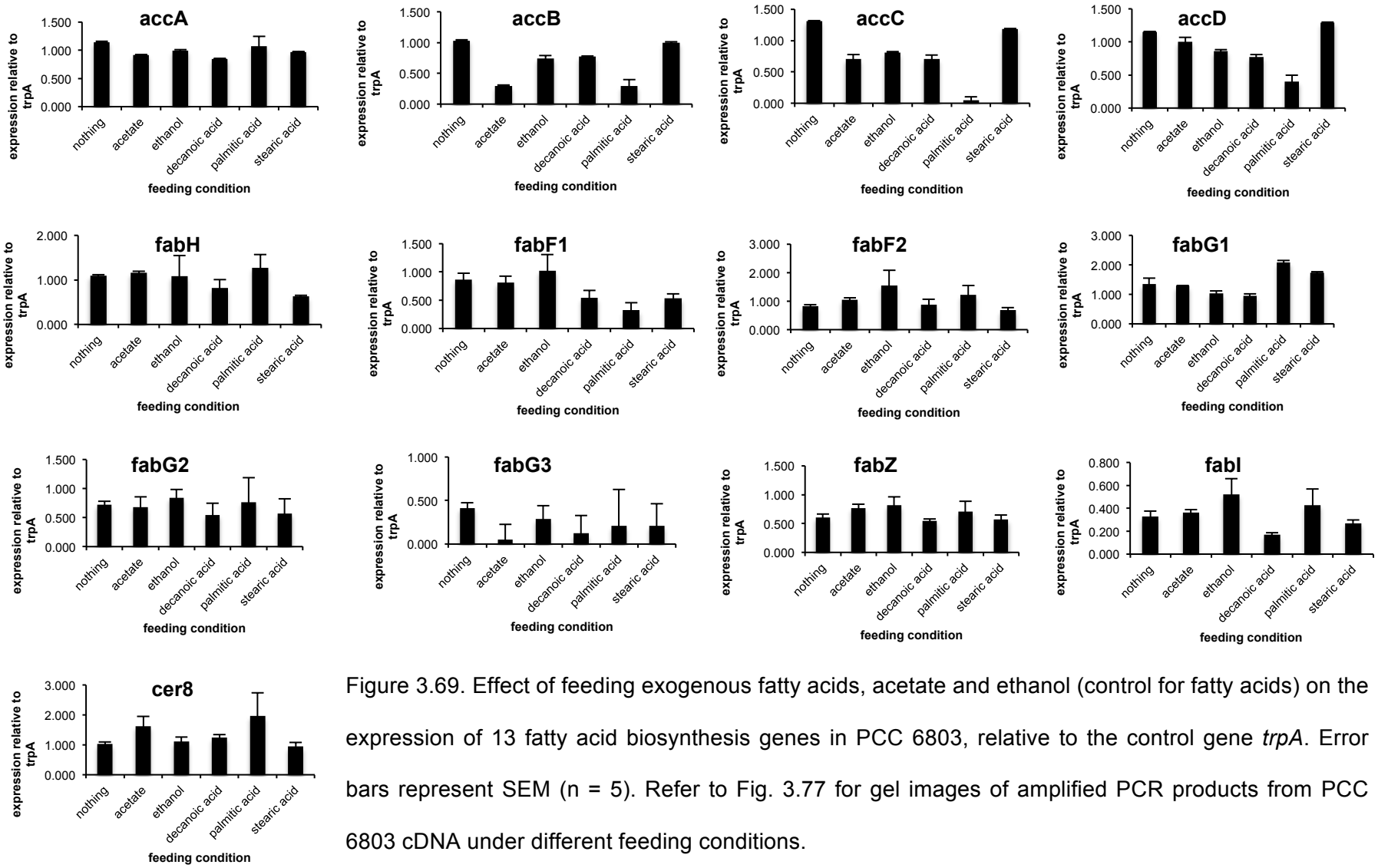


Figure 3.69. Effect of feeding exogenous fatty acids, acetate and ethanol (control for fatty acids) on the expression of 13 fatty acid biosynthesis genes in PCC 6803, relative to the control gene *trpA*. Error bars represent SEM (n = 5). Refer to Fig. 3.77 for gel images of amplified PCR products from PCC 6803 cDNA under different feeding conditions.

Figure 3.70. Sample DNA gel images of bands of PCR products of 13 fatty acid biosynthesis transcripts plus control gene *trpA*, amplified from cDNA of PCC 6803 that had been fed a range of fatty acids, acetate and ethanol from which the data in Fig. 3.70 was derived.

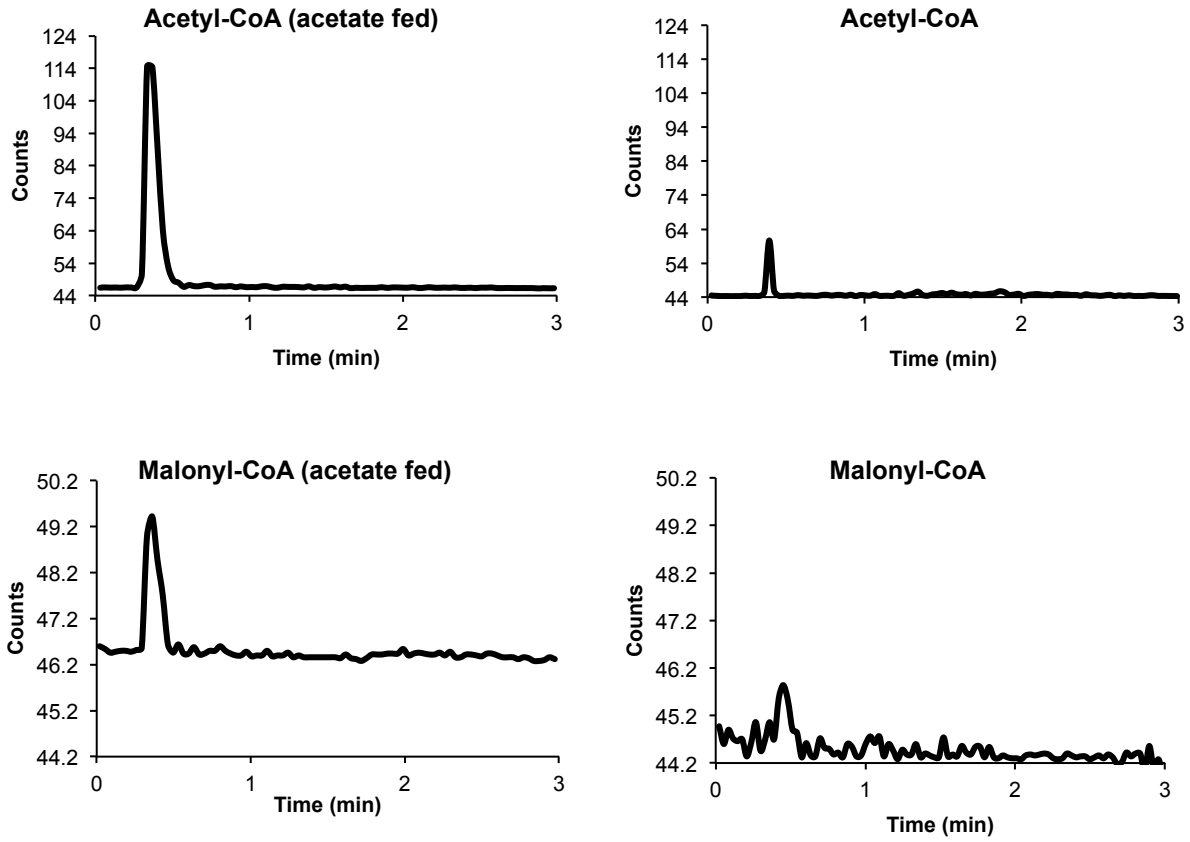


Figure 3.71. Concentrations of acetyl-CoA and malonyl-CoA are increased in extracts from PCC 6803 when acetate is fed for 24 h prior to harvesting.

3.4.3 Effects of fatty acid feeding on the efficiency of fatty acid biosynthesis

In order to determine whether this down-regulation observed in fatty acid biosynthesis genes results in a reduced efficiency of fatty acid biosynthesis, PCC 6803 cultures were fed acetate, decanoic acid, palmitic acid and stearic acid as before, along with a control sample where there was no feeding. After 24 h, these cultures were spiked with 1,2-^{14}C-acetate for 4 h. After this period, cells were harvested, extracted and fractionated into culture medium, aqueous phase and lipid phase as described in 2.9.2 in order to determine to what extent the cultures were able to incorporate acetate into fatty acids.

Most (>97 % of total radioactivity from label) of the acetate label remained in the culture medium (Table 3.21). Cyanobacteria are not thought to have a specific uptake mechanism (Gibson, 1981) unlike with fatty acids, using acyl-ACP synthetase (encoded by the *cer8* homolog) to mediate import of fatty acids (Von Berlepsch et al. 2012). However much higher levels of acetate import have been observed previously in other studies, when acetate was fed to other strains of cyanobacteria from *Synechococcus* and *Aphanocapsa* (Ihlenteldt and Gibson, 1977). Ihlenteldt and Gibson found that under conditions of CO_2 starvation, acetate uptake was reduced – the experiments conducted in this study were at ambient levels of CO_2, this could possibly account for the low level of acetate uptake. Furthermore, less acetate was taken up by cultures that had been fed decanoic acid and palmitic acid previously.

Cultures that were fed decanoic acid and palmitic acid incorporated significantly less acetate label into lipids than the control culture and cultures that were fed stearic acid (Table 3.21), 2 sample t tests return $p < 0.0001$, t = 19.1603 and 20.373 respectively versus control. This shows that inhibition of the expression of fatty acid biosynthesis genes *accB, accC* and *accD* by palmitic acid feeding, and inhibition of *fadI* expression by decanoic acid feeding results in a down-regulation of the activity of the fatty acid biosynthetic pathway. Stearic acid showed a less pronounced, but still significant

reduction in the level of incorporation of acetate into lipids (p = 0.0015, t = 8.7207), possibly due to its low solubility.

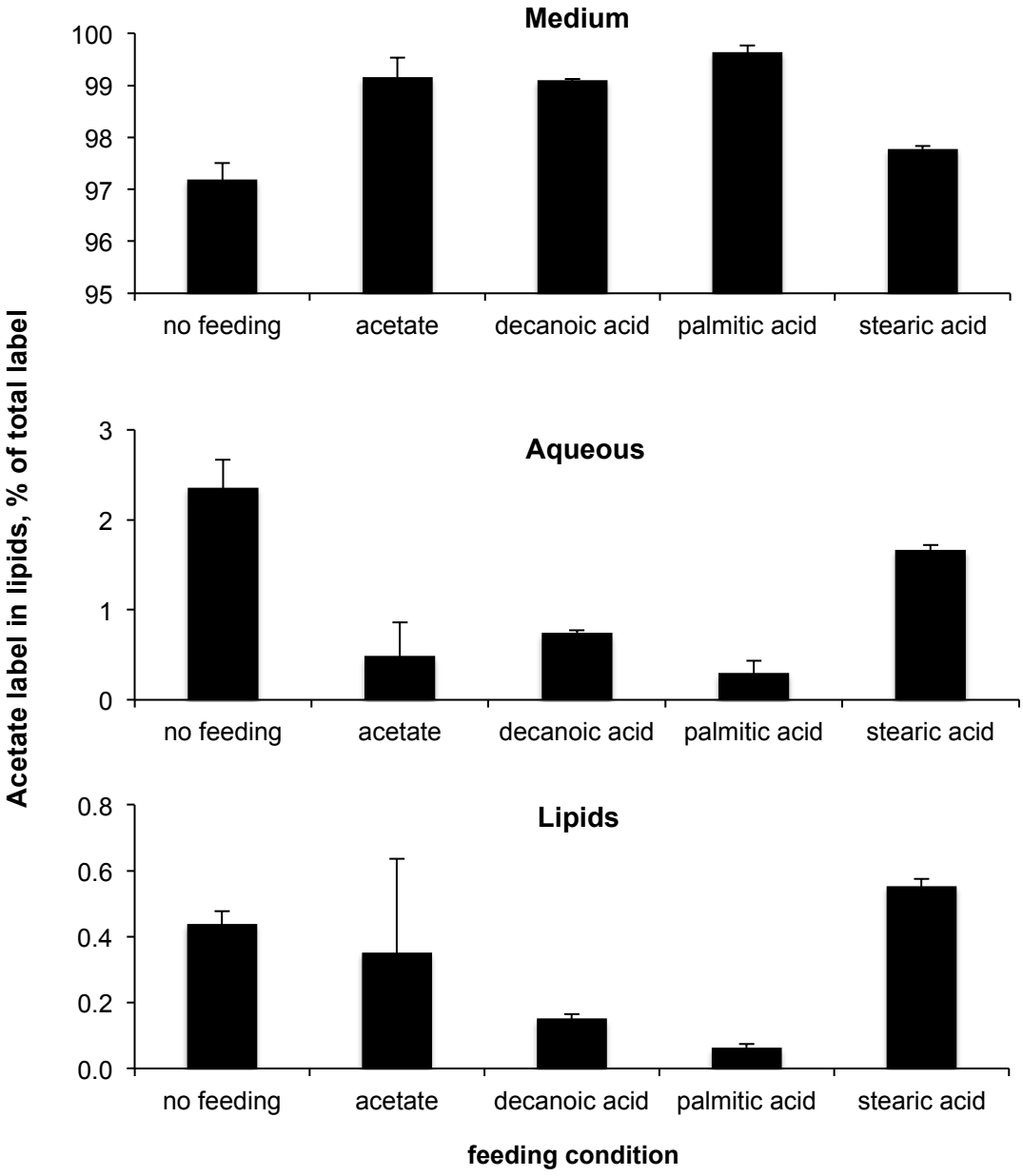


Figure 3.72. Average % of total label from 1,2-^{14}C-acetate, fed to PCC 6803 that was incorporated into culture medium, aqueous phase, lipid phase after being fed acetate, decanoic acid, palmitic acid and stearic acid prior to labelling. ± SEM (n = 3).

3.4.5 Discussion

Much work has been conducted on the regulation of fatty acid biosynthesis in a range of bacteria (Fujita et al. 2007). Studies on the metabolic regulation in cyanobacteria have been somewhat limited, the major pathway other than photosynthesis being the regulation of nitrogen metabolism (Merida et al. 1991; Muro-Pastor and Florencio, 2003; Alfonso et al. 2001). Additionally other cyanobacterial metabolic pathways, such as sugar metabolism (Osanai et al. 2005; Osanai et al. 2006) have been studied. The regulation of lipid metabolism in cyanobacteria has been limited to the mechanisms of fatty acid desaturation in response to temperature stress (Los and Murata 1997; Murata, 1989) and other forms of stress such as desiccation, salt stress, photoinhibition and peroxidation (Singh et al. 2002). However to date no studies have been conducted on cyanobacterial fatty acid biosynthesis and metabolism.

Little work has been carried out on what happens to the regulation of fatty acid biosynthesis under conditions of excess fatty acids, as it is known that in other organisms that β-oxidation is upregulated; therefore most work on fatty acid metabolism focuses on what happens to fatty acid metabolism under carbon-limiting conditions of starvation, such as the stringent response in bacteria (Magnusson et al. 2005; Battesti and Bouveret, 2006; Chatterji and Ojha, 2001). This is the first time to date that the regulation of fatty acid biosynthesis has been studied in cyanobacteria. It has been shown that a form of negative-feedback regulation resulting from the presence of excess fatty acids occurs, acting at least at the transcriptional level, as has been shown by the RT-PCR experiments conducted in this chapter, and that this feedback inhibition results in a reduction in the level of fatty acid biosynthesis. Whether this feedback inhibition is the result of the fatty acids directly or results from a downstream metabolite of fatty acids is not known.

It is clear that palmitic acid results in the greatest effect on the inhibition of fatty acid biosynthesis, inhibiting *accB*, *accC* and *accD* of the acetyl-CoA carboxylase complex. However at the same time the expression of acyl-ACP synthetase, *cer8*, also termed *aas,* is upregulated, this allows the cyanobacteria to preferentially take up free fatty acids. This idea makes sense in terms of energy budgets, as it takes less energy to take up free fatty acids as opposed to synthesising them *de novo*. Acyl-ACP synthetase is responsible for import of exogenous fatty acids across the cell membrane (Von Berlepsch et al. 2012), as well as taking a part in recycling free fatty acids that are generated by hydrolysis of membrane lipids by to acyl-ACPs (Kaczmarzyk and Fulda, 2010). This upregulation observed in *cer8* upon feeding of fatty acids could also account for the background activity of apparent acyl-CoA synthetase activity on palmitic acid that was observed in 3.2.6. Liu et al. (2011) exploited the fact that acetyl-CoA carboxylase appears to be the rate-controlling enzyme, having observed the same response in *E. coli* (Davis et al. 2000). They attained high levels of free fatty acid production in *Synechocystis* by overexpressing acetyl-CoA carboxylase, along with the deletion of *cer8*, as well as a host of other genetic modifications, such as introduction of thioesterases from the plants *Umbellularia californica*, *Cuphea hookeriana* and *Cinnamomum camphorum,* under control of the promoters of highly expressed RuBisCO carboxylase and phycocyanin synthetase genes, as well as deletion of genes including those involved in PHB synthesis and peptidoglycan assembly. If this group had known the mechanisms of the regulation of fatty acid biosynthesis, perhaps a greater degree of control of the acetyl-CoA carboxylase complex could have been achieved, resulting in even higher yields of free fatty acid secreted.

Decanoic acid appeared to inhibit the transcription of a different enzyme to that of palmitic acid, FabI*,* instead of the ACCase complex. *fabI* encodes for an enoyl-ACP reductase, that reduces the carbon-carbon double bond found between carbons 2 and 3 of its enoyl-ACP substrate, resulting in an acyl-ACP product. Why the presence of decanoic acid specifically reduces the transcription rate of this enzyme is not clear;

perhaps a different enzyme utilizes the decenoyl-ACP substrate of this enzyme, although possible candidates for as to what this enzyme could be remain elusive. Enoyl-CoA hydratases utilize enoyl-CoAs for PHA biosynthesis (Park and Lee, 2003) and it is possible that an ortholog in PCC 6803 could utilize decenoyl-ACP as its substrate, however as was shown in 3.1.2, the closest match to this enzyme was a 1,4-dihydroxy-2-naphthoyl-CoA synthase, encoded by *menB,* that had been characterized by Song and Guo, 2012 and is involved in vitamin K biosynthesis. The apparent inhibition caused by the feeding of acetate was probably caused by a downstream product that acetate is a precursor of, as was described in 3.4.2.

As most cyanobacteria do not possess any significant matches to the transcriptional regulatory proteins of fatty acid biosynthesis and metabolism that are found in bacteria (with the exceptions of PCC 7425 and MBIC 11017), the regulatory protein(s) that modulates the expression of fatty acid biosynthesis must be somewhat novel. Additionally the fatty acid biosynthetic genes in PCC 6803 are not organized into operons as has been observed in some other bacteria, for example *B. subtilis*, where a *fabHA-fabF* operon and *fapR-plsX-fabD-fabG* operon are present (Schujman et al. 2003), however it's worth noting that these genes are not under the control of an operon in *E. coli* either. In order to elucidate this regulator, it would be necessary to search for conserved sequences upstream of the fatty acid biosynthetic genes that are found to be regulated, namely *accB*, *accC* and *accD* to which the regulator would bind, causing repression of transcription of these genes. In *B subtilis* there are *cis*-sequences to which the FapR protein binds (Schujman et al. 2006). In *E. coli* a 17 bp FadR binding sequence 40 bp upstream of *fabA* and *fabB* (Henry and Cronan, 1992) for the activation of these genes by assisting the recruitment of RNA polymerase, as well as larger binding sequences between -30 and +10 of the start sites of *fad* genes where repression occurs by the blocking of binding of RNA polymerase.

Experimental evidence for the interaction of these DNA sequences and the target protein could be sought by performing gel retardation assays, isolating the interacting

protein, purifying it and it sequencing using nano-LC-ESI-QToF-MS. Characterisation of the target enzyme could be attained with the use of knock-outs of the enzymes' coding gene, utilizing homologous recombination to introduce an antibiotic resistance cassette in place of the target gene and the selecting for organisms with the knocked out gene by growing in the presence of the desired antibiotic, although as stated in 3.3.7, PCC 6803 can have up to 12 copies of its chromosome (Labarre et al. 1999), which can make creating fully knocked out cell lines a time-consuming process. Determination of the mechanism of the regulatory proteins' operation could be achieved by using the method described above, coupled to RT-PCR. If the gene were expressed at a much lower level in knockouts, then it would mean the regulator operates by promotion, whereas if a high level of constitutive expression was observed it would mean that the regulator operates through repression.

In order to discover potential agonists or antagonists of this regulatory protein, the gel retardation assays could be performed in the presence of candidate compounds that are likely to be (ant)agonists of the regulator, such as free fatty acids, acyl-ACPs, malonyl-CoA or other downstream metabolites of fatty acids or acetate. Compounds affecting the binding of the protein to the DNA sequences would give different results to those that do not affect the binding.

Another question is whether direct inhibition of the fatty acid biosynthesis enzymes occurs as a more rapid response to excess fatty acids. Evidence for direct inhibition of fatty acid biosynthetic enzymes could be sought by performing enzyme assays on the PCC 6803 acetyl-CoA carboxylase complex (Nikolau et al. 1981; Howard and Ridley, 1989; Kroeger et al. 2011), along with the varying concentrations of potential inhibitors such as fatty acids, acyl-ACPs, malonyl-CoA or other possible candidate compounds.

4. Discussion

The initial aim of this project was concerned with investigating the optimization of metabolic pathways in cyanobacteria in order to improve the yield of commercially important hydrocarbons that many of these organisms naturally synthesise. The main candidate pathway for study was β-oxidation. One thing that was striking was that nearly all cyanobacteria with sequenced genomes appeared to completely lack the β-oxidation pathway according to genome annotation based on homology.

It is generally assumed that fatty acid degradation is universal, occurring in all organisms. The apparent lack of β-oxidation genes according to annotation led to the formulation of the initial hypothesis of the project; that cyanobacteria lacked an active fatty acid degradative metabolic pathway. This poses a problem, as the basis of the project was to prove a lack of existence of this pathway. In order to demonstrate this, multiple streams of empirical evidence were sought. These streams of empirical evidence consisted of bioinformatic analyses, acyl-CoA assays, acyl-CoA dehydrogenase and oxidase assays, metabolism of radiolabelled fatty acids and ectopic expression of β-oxidation genes in cyanobacteria, as well as PCR and RT-PCR for *Acaryochloris*.

Initial bioinformatic investigations revealed that potential cyanobacterial protein sequences with similarity to β-oxidation enzymes were probably involved in other metabolic pathways rather than β-oxidation or were not present at all. As acyl-CoAs, the substrates of β-oxidation were not detected; the first piece of empirical evidence demonstrating that β-oxidation is not present in cyanobacteria was obtained. Further evidence for the lack of acyl-CoAs and β-oxidation was sought by assaying two enzymes that use acyl-CoAs as a substrate; acyl-CoA dehydrogenase and acyl-CoA oxidase. No activity for either enzyme in cell-free extracts could be detected. It could be argued that β-oxidation, or another form of perhaps novel fatty acid degradation that

didn't require the presence of acyl-CoAs was occurring in cyanobacteria. Therefore, a third experiment examining the ability of cyanobacteria to release CO_2 from fatty acids, or convert them into other compounds resulting from their degradation, was performed. This provided more evidence that cyanobacteria were unable to degrade fatty acids, but instead used them as structural molecules, integrating them into their thylakoids, as could be seen from the large amounts of glycolipids that incorporated these fatty acids. This argument makes sense if the large amount of thylakoids that cyanobacteria possess and their essential function for photosynthesis is taken into account. However it could still be argued that cyanobacteria do not degrade fatty acids under those specific conditions, possibly needing parameters that could be difficult or impossible to achieve with the experimental setups used in this project.

Despite the evidence presented here, it is still difficult to say with certainty that cyanobacteria do not degrade fatty acids. A lack of evidence does not necessarily mean that something does not exist; it could simply mean that it has not yet been found. For example Knoop hypothesized the presence of fatty acid degradation occurring via attack on the β carbon of the fatty acid in 1904, before that point it was not known that fatty acids were degraded in this manner. Up until 2011 it was also assumed that the cyanobacteria had an incomplete TCA cycle, as two separate groups in 1967 failed to detect α-ketoglutarate dehydrogenase activity (Pearce and Carr, 1967; Smith et al. 1967), a key enzyme in this metabolic pathway responsible for conversion of α-ketoglutarate to succinyl-CoA. However Zhang and Bryant (2011) reported that this step of the TCA cycle instead proceeds *via* a novel enzyme, α-ketoglutarate decarboxylase, yielding succinic semialdehyde instead of succinyl-CoA, which is then converted to succinate by the novel succinic semialdehyde dehydrogenase, as opposed to succinyl-CoA synthetase found in most other organisms. This study highlights the consideration that needs to be taken when analyzing negative results. Another thing worth noting from the above report is that cyanobacteria avoid using

another coenzyme A bound metabolic intermediate and lack yet another enzyme that has CoA synthetase activity.

Chapter 3.2 saw the introduction of an element of β-oxidation into strain PCC 6803, with ectopic expression of *fadD* from *E. coli*. The data obtained in this chapter indirectly provides further evidence of the lack of β-oxidation in cyanobacteria, as it shows that it is possible to introduce acyl-CoA synthesis in cyanobacteria and gives validation of the acyl-CoA assay.

Introduction of acyl-CoAs may also be beneficial from an engineering perspective for the production of other compounds of commercial value such as fuels and chemicals (Fig. 4.1). For example if *fadD* expressing PCC 6803 was engineered with an exogenous acyl-ACP thioesterase, resulting fatty acids could be channeled into acyl-CoAs in a controlled manner if these genes were under the control of inducible promoters. These acyl-CoAs could serve as precursors to desired compounds with less impact on essential endogenous biochemical pathways that are essential to the organisms' survival. Strain PCC 7937 already has endogenous thioesterases, acting upon 12:0-ACP's to 18:0-ACP's, as well as being diazotrophic and so not having a nitrogen requirement for growth, meaning that this strain could be the ideal candidate for this purpose.

Similar alterations of metabolic pathways have been shown in other organisms, for example the reversal of the β-oxidation pathway for the production of butanol, long chain fatty alcohols and hydroxyl- and keto- fatty acids (Dellomonaco et al. 2011). This was achieved in *E. coli* by constitutive expression of β-oxidation and thioesterase genes, by knocking out regulators FadR, AtoC and ArcA and replacing the cAMP receptor protein with a cAMP independent mutant. The acyl-CoA dehydrogenase encoded by *ydiO* that operates under anaerobic conditions was found to be reversible, unlike the irreversible enzyme encoded by *fadE* that operates under aerobic conditions, therefore this was overexpressed. Finally, termination enzymes encoded by *fucO* to

yield *n*-butanol, *tesA* to yield 3 hydroxybutyric acid and *tesB* to yield 3-ketobutyric acid were overexpressed. In order to achieve longer chain products, *fadA* that encodes a thiolase, which under these conditions operates in reverse, was overexpressed. Reversal of the β-oxidation pathway only operated if no fatty acids were present in the culture medium.

Although introduction of a reversed β-oxidation cycle into cyanobacteria would be possible, this would be difficult as no endogenous β-oxidation genes are present, so *E. coli* genes would need to be ectopically expressed in the same manner as *fadD,* and may not work as efficiently as in their host organism. However the genes encoded by *fadA* and *fadJ* may be of use for the synthesis of precursors of β-ketoacids and hydroxyacids that are of use for pharmaceuticals, cosmetics, waxes and in the food industry (Fig 4.1).

Other commercially important compounds that could be derived from acyl-CoAs include fatty alcohols that can be used as fuels, detergents, waxes, cosmetics and in the food industry. Tan et al. (2011) expressed fatty acyl-CoA reductases from *A. thaliana,* jojoba and mice in PCC 6803 to produce fatty alcohols. Note however that this strain did not have *fadD* and therefore no acyl-CoAs, so it appears that these fatty acyl reductases may also be able to accept acyl-ACPs as their substrate, as has been observed with acyltransferase and 1-acyl-sn-glycerol-3-phosphate acyltransferase that ligate fatty acids into the glycerol backbones of fatty acids (Wilkison et al. 2000). The *ole* genes, responsible for the biosynthesis of alkenes in a range of bacteria (Sukovich et al. 2010) also use acyl-CoAs as their precursors, synthesising these compounds via head-to-head condensation as opposed to acyl-ACP reductase and aldehyde decarbonylase in cyanobacteria. The *oleA* gene product performs the condensation reaction, forming a ketone and the remaining 3 enzymes, encoded by *oleB, oleC* and *oleD* reduce this to an alkene. The pathway could either be arrested after the condensation to yield ketones, useful for solvents, pharmaceuticals and polymer

precursors, or allowed to proceed to form alkenes that can be used as fuels, lubricants, surfacing and as polymer precursors (Fig. 4.1). However these condensing enzymes may also be able to accept acyl-ACPs as well as acyl-CoAs (Beller et al. 2010). If the intracellular pool of acyl-CoAs is much greater than that of acyl-ACPs, then the effect of broad range substrate specificity will not be such an issue as the acyl-CoAs will outcompete the acyl-ACPs for the enzymes' active sites. Furthermore the synthesis of hydrocarbons via this method are not infringing on the patents taken out by the biofuel company LS9 on the use of cyanobacterial acyl-ACP reductase and aldehyde decarbonylase. Carbon could even be diverted away from this pathway by knock-out of these genes.

If these modifications were coupled with those that were performed by Liu et al. (2011), specifically the deletion of the *aas* gene, encoding acyl-ACP synthetase that is responsible for recycling free fatty acids (Kazcamarzyk et al. 2010) and of PHA biosynthesis genes, then a cyanobacterial strain that gives high yields of these desired products may be possible.

Understanding the regulatory mechanisms behind fatty acid biosynthesis will also aid in controlling the flux of carbon into the useful pathways described above. Some of this work was carried out in chapter 3.4. If cyanobacteria do indeed lack the ability to degrade fatty acids, then it is reasonable to suppose that their *de novo* synthesis must be very highly regulated. Little research had been conducted on the regulation of fatty acid biosynthesis in cyanobacteria, most focusing on heterotrophic bacteria. It was found that like heterotrophic bacteria, the expression of fatty acid biosynthetic genes was modulated at the transcriptional level and that repression occurred from fatty acid feeding, although inhibition itself was probably by resulting downstream metabolites of fatty acids. There is also the possibility of regulation occurring by direct feedback inhibition acting upon fatty acid biosynthesis enzymes.

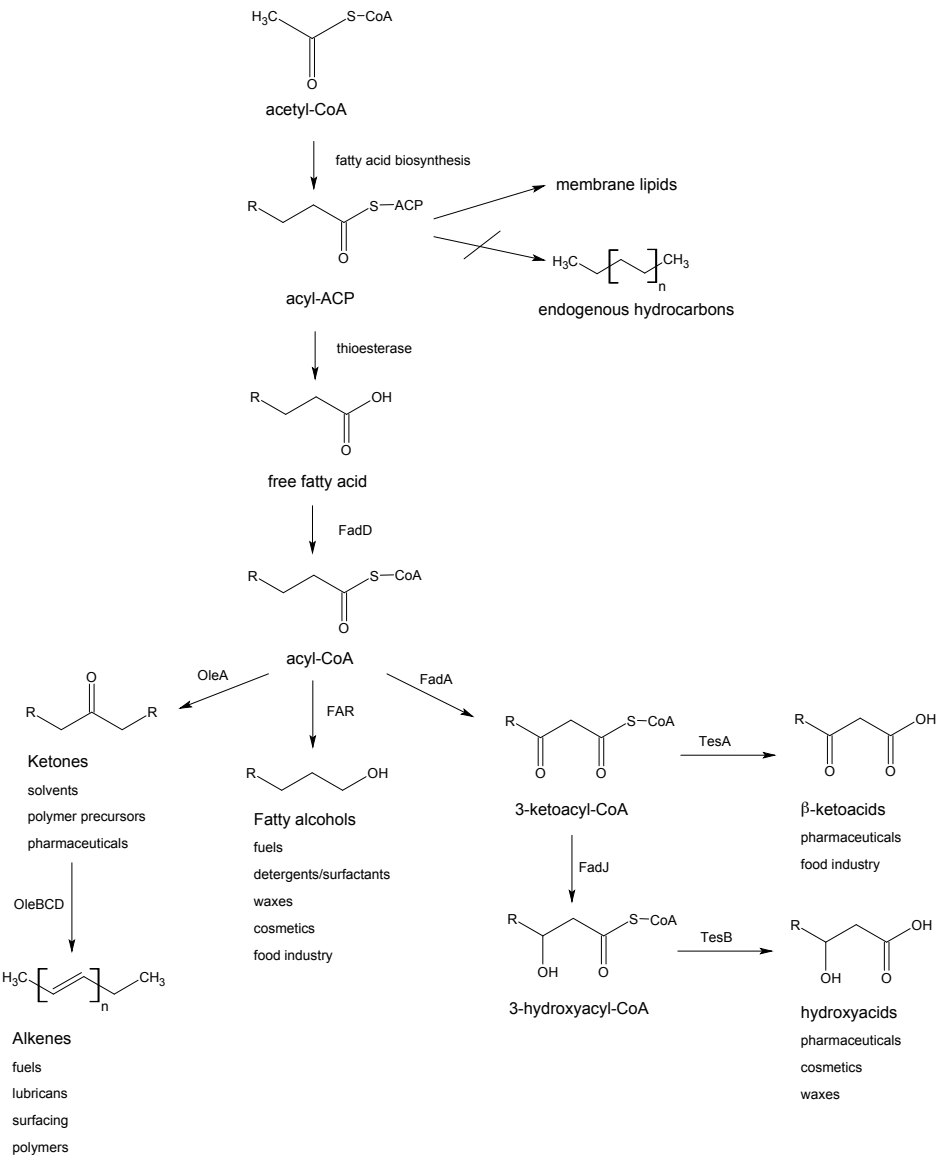


Figure 4.1. Possible applications for acyl-CoAs in cyanobacteria. These include β-ketoacids, hydroxyacids, fatty alcohols, ketones and alkenes. Acetyl-CoA is converted into acyl-ACP via the fatty acid biosynthetic pathway. Thioesterases release ACP from the acyl moiety to allow acyl-CoA synthetase (FadD) to make acyl-CoAs. Acyl-CoAs can then be converted into desired compounds by a range of enzymes working together. OleA – acyl-CoA condensing thiolase; OleBCD – hydrolase, thiolase and dehydrogenase involved in steps of alkene synthesis; FAR – fatty acyl-CoA reductase; FadA – acetyl-CoA acyltransterase; FadJ – 3 hydroxyacyl-CoA dehydrogenase; TesA – acyl-CoA thioesterase I; TesB – acyl-CoA thioesterase II.

Another area of metabolism that could be limiting to acyl-CoA production is coenzyme A synthesis itself. CoA is synthesised from a series of 6 reactions from precursors β-alanine and pantoate as described in 1.6.1. This pathway may require upregulation in cyanobacteria in order to ensure there is a large enough CoA pool to accept the acyl chains.

Although by genome annotation most of the cyanobacteria appeared to lack the genes required for β-oxidation, it appeared that two strains, MBIC 11017 and PCC 7425 did have all the required genes. Although these two strains were unobtainable, a closely related strain, HICR111A was, so analyses to determine whether fatty acid degradation occurred in this organism was performed, the result again was negative. One criticism of performing the analyses on this strain is that the other two strains could be substantially divergent enough from this one that they may have a functional β-oxidation pathway, although 16S sequencing conducted by Mohr et al (2010) shows that they are very closely related. Another criticism of the analyses of this strain lies in the RT-PCR that was performed that demonstrated that the genes of β-oxidation were not transcribed. It is a possibility that no transcripts were detected due to either the PCR conditions not being optimal, or that the genes are simply not transcribed under the specific conditions that the cyanobacterial strain was cultured in. It is known that some *Acaryochloris* strains have a symbiotic relationship with other marine organisms such as tunicates (Lopez-Legentil et al. 2011); perhaps it is under these currently near impossible to replicate conditions that gene expression of β-oxidation is induced.

From these data obtained in this project, it seems that fatty acids probably play a more structural role in the cell as opposed to an energy storage role that is observed in many other organisms. It appears that the task of energy storage is probably down to glycogen and PHA's that both share fatty acids' properties of being water insoluble and so have minimal impact on the cells osmotic potential. PHA's are also highly reduced molecules like fatty acids, and as such are also classed as lipids, releasing a high net

yield of energy upon oxidation (Dias et al. 2008). PHA biosynthesis and accumulation has been reported in a range of cyanobacteria (Hein et al. 1998; Miyake et al. 2000). PHA's are also commercially important compounds with a range of applications, primarily in the area of bioplastics (Philip et al. 2007). The two model organisms for the study of PHA metabolism are *A. vinosum* and *R. eutropha*, which are members of the β- and γ-proteobacteria. Most of the bacteria that can accumulate PHA's are from these classes of organisms.

As well as cyanobacteria sharing the metabolic traits of PHA synthesis with some other bacteria, they also may share a lack of an active β-oxidation pathway. Simple searches of the genomes of a range of organisms reveal that many other organisms may also lack this pathway; at least 31 genera of proteobacteria, 12 genera of firmicutes, 5 genera of bacteroidetes, *Chlorobaculum* and *Chlorobium* of the green sulphur bacteria, *Treponima* and *Spirochaeta* of the Spirochaetes, *Bifidobacterium* and *Terriglobus*, 3 genera of Archaea, 2 genera of protists and even 1 animal, the parasitic flatworm *Schistosoma mansoni,* all lack the genes required for β-oxidation according to KEGG.

Many of these species are chemoheterotrophic anaerobes, such as *Thiobacillus denitificans, Sulfurimonas autotrophica* and *Desulfovibrio*, whist *Chlorobaculum* and *Chlorobium* are photoautotrophic anaerobes that use sulphide ions, hydrogen or ferrous iron as their electron donors as opposed to water in oxygenic photosynthesis. Other bacterial strains lacking annotated β-oxidation genes have unusual methods of metabolism, for example methylotrophs such as *Methylococcus capsulatus* and *Methylobacillus flagellatus.*

Other anaerobic bacteria lacking β-oxidation genes are found living in the gastrointestinal tract of animals, such as *Bacteroides*, the major component of human gut microflora, as well as *Lactobacillus*, *Enterococcus, Clostridium* and *Bifidobacterium* that are minor constituents (Simon and Gorbach, 1982). Additionally the cellulose-degrading rumen gut bacterium *Fibrobacter succinogenes* (Koike et al. 2004) also

lacks β-oxidation genes. These species may have lost their capacity for fatty acid degradation as they are able to obtain a constant supply of carbon sources from the gastrointestinal tract. Some strains of pathogenic bacteria, such as *Pasteurella mutidocida*, *Campylobacter*, *Bartonella, Listeria* and *Clostridium* may have lost β-oxidation genes for the same reason. A lack of β-oxidation in these organisms could potentially be useful with regards to drug development, particularly if they utilise PHA's for energy storage, although this could negatively affect many of the commensal bacteria also mentioned above. Furthermore, two parasitic protists of the genus *Entamoeba* and the flatworm *Schistosoma mansoni* lack β-oxidation genes, again probably having lost them as a by-product of their parasitic lifestyles. In fact, *S. mansoni* has been found to lack a fatty acid biosynthetic pathway also, utilising its hosts' fatty acids instead (Meyer et al. 1970).

Organisms from the domain of Archea, such as *Methanococcus, Methanocaldococcus* and *Thermocrinus* also lack β-oxidation genes, as they have different types of lipid compared to the bacteria and eukarya, using ether-linked polar membrane lipids that have isoprenoid chains as opposed to fatty acids (Koga and Morii, 2007). As the archea use such different lipids, it is likely they never evolved a β-oxidation pathway. This raises an interesting question in that did cyanobacteria lose the ability to degrade fatty acids as part of their lifestyle in a similar way to commensal and pathogenic organisms, i.e. the fact that they always had a reliable source of energy in the form of light, for which fatty acids were required structurally as part of thylakoid membrane lipids, meaning that they could dispense with this mechanism; or was it that the ancient cyanobacteria simply never evolved fatty acid degradation in the first place? There is phylogenomic evidence that β-oxidation can be traced back to ancestors of the δ proteobacterial species of *Myxococcus* (Schluter et al. 2011). They go on to suggest that these genes were distributed to some bacteria by horizontal gene transfer,

including a mitochondrial ancestor, and that these genes were transferred to eukaryotes by endosymbiotic gene transfer.

A possible biochemical explanation for the lack of β-oxidation in anaerobic organisms stems from the requirement for the coenzyme NAD^+ that is reduced by glyceraldehyde-3-phosphate dehydrogenase that oxidises glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate to form NADH + H^+. During anaerobic metabolism the NAD^+ must be regenerated by transferring its electrons to an electron acceptor. These electron acceptors can be inorganic substances such as sulphate ions as in *Desulfovibrio*, or pyruvate as in *Lactobacillus* to produce lactate. The regenerated NAD^+ can then act as an oxidising agent for glyceraldehyde-3-phosphate. In β-oxidation, the dehydrogenation of 3-hydroxyacyl-CoA to 3-ketoacyl-CoA by 3-hydroxyacyl-CoA dehydrogenase also generates NADH and H^+ from NAD^+ for every turn of the cycle, meaning that for longer chain fatty acids lots of NAD^+ is used up, causing an imbalance and meaning that conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate cannot happen. As glycolysis is the anaerobic cells' only means of producing energy, this would result in death of the organism. It has been shown that some strains of cyanobacteria are capable of anoxygenic photosynthesis and growth, utilising sodium sulphide (Garlick et al. 1977) and hydrogen (Belkin and Padan, 1978) as electron donors.

Finally, it could be that hydrocarbon biosynthesis could act as a sink for excess fatty acids, in the case of them accumulating to toxic levels they could be converted to chemically inert hydrocarbons (alkanes) and then secreted. Hydrocarbons have been reported in many other bacteria (Davis, 1967; Albro, 1970; Sukovich et al. 2010), although it appears the biosynthetic pathway is somewhat different. As stated above, hydrocarbons in other bacteria are formed by head to head condensation of acyl-CoAs or acyl-ACPs (Beller et al. 2010) as opposed to the acyl-ACP reductase and aldehyde decarbonylase observed in cyanobacteria, the genes of which are lacking according to

BLAST searches of the genomes of these organisms. A lack of fatty acid degradation may enhance cyanobacteria's commercial value with regards to the production of biofuels.

5. Appendix

5.1 Growth curves

In order to determine the rate of growth of the three initial cultures of cyanobacteria to be investigated, strains PCC 6803, PCC 7120 and PCC 7937, growth curves were established by measuring the optical density at 730 nm (OD_{730nm}) (Fig. 5.1). This type of preliminary experiment was useful because it gives an approximate idea of when a culture will reach a specific OD or growth phase and for determining the length of viability of a culture. The experiment was conducted over a period of 29 days, after this period PCC 6803 was observed to enter death phase, the culture starts to turn yellow and a simultaneous drop in $OD_{730\ nm}$ was observed (data not shown). Strains PCC 7120 and PCC 7937 were able to remain in stationary phase for a longer period of about 50 days. PCC 6803 grew initially at a higher rate than PCC 7120 and PCC 7937, but entered stationary phase at a lower $OD_{730\ nm}$ of about 10 to 11. Growth rates and dynamics of PCC 7120 and PCC 7937 were very similar, PCC 7120 growing at a slightly higher rate initially. Distinct lag, exponential and stationary phases were apparent. Lag phase lasted approximately 1 day for all strains, PCC 6803 was in exponential phase for 7 days between an approximate $OD_{730\ nm}$ of 2 to 10, PCC 7120 and PCC 7937 remains in exponential phase for 13 days between an approximate $OD_{730\ nm}$ of 2 to 14.

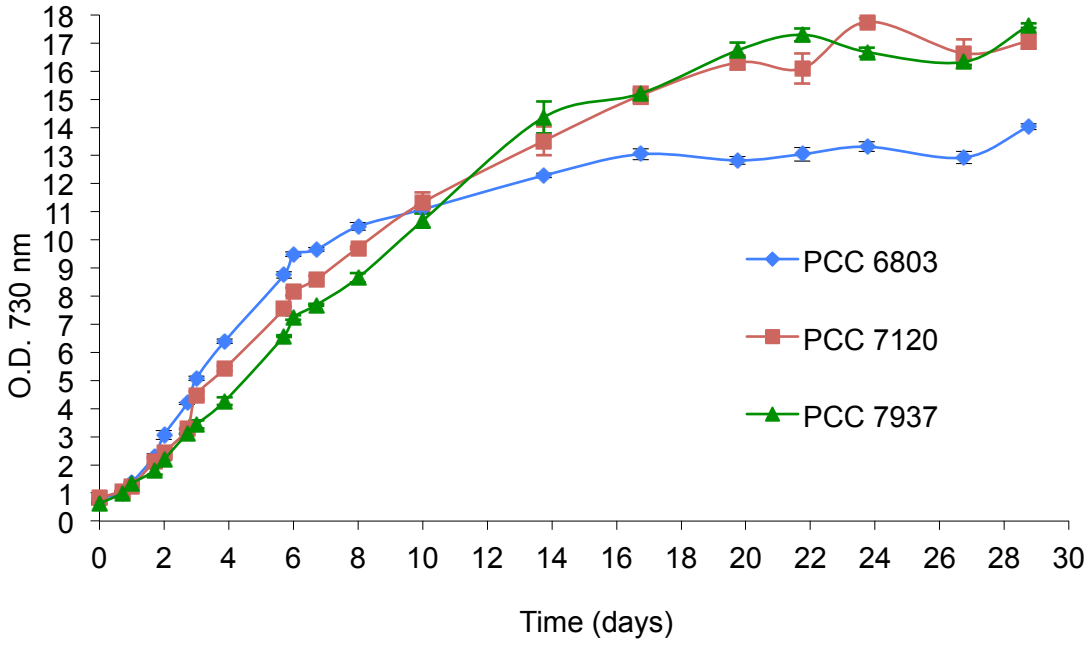


Figure 5.1 Growth curves of PCC 6803, PCC 7120 and PCC 7937. ± S.E. n=3.

5.2 Feeding of fatty acids to cyanobacteria

A range of solvents were tested to determine if they could 1. dissolve stearic acid and 2. their effects on cyanobacteria after incubation with PCC 6803 and PCC 7120 for 24 h.

1% ethanol was able to partially dissolve stearic acid, appears to have minimal effect on the viability of cyanobacteria (Table 5.1) and is the least costly out of all the tested solvents, this was deemed the most suitable to use as a solvent, observed solubility of this solvent is in line with data published by Ralston and Hoerr, 1942.

PCC 6803 and PCC 7120 were fed fatty acids in 1 % v/v ethanol for 24 h at a range of concentrations between 0.1 mM to 5 mM. It was found that unsaturated fatty acids (i.e. oleic acid) at high concentration (5 mM) killed the cyanobacterial culture (Fig. 5.2). This does not happen when feeding saturated fatty acids. High concentrations of

unsaturated fatty acids in membrane lipids can lead to peroxidation and breakdown of cell membranes (Schnitzer et al. 2007).

Table 5.1 Effects of various solvents on cyanobacteria and their ability to dissolve stearic acid. * these solvents are completely insoluble in water and form a layer that sits on top of the medium.

Solvent	**Able to dissolve 1 mM Stearic acid?**	**Observed effect on cyanobacterial cultures**
Water	No	None
DMSO 10% in water	No	None
Ethanol 10% in water	Yes	Culture dies: blue pigment observed in supernatant after centrifugation – possibly phycocyanin
Ethanol 5% in water	Yes	
Ethanol 1% in water	Yes	None
Isobutanol*	Yes	Culture dies: turns an orange-brown colour
1-pentanol*	Yes	
1-octanol*	Yes	
Ethyl acetate*	Yes	
Acetone 1% in water	Yes	Culture dies: green pigment observed in supernatant after centrifugation – possibly chlorophyll
Methanol 1% in water	Yes	
Hexane*	Yes	Hexane had evaporated after 24 h; ppt of stearic acid in medium remains
Nonane*	Yes	None
Decane*	Yes	None
Tetradecane*	Yes	None
Tetradecene*	Yes	None

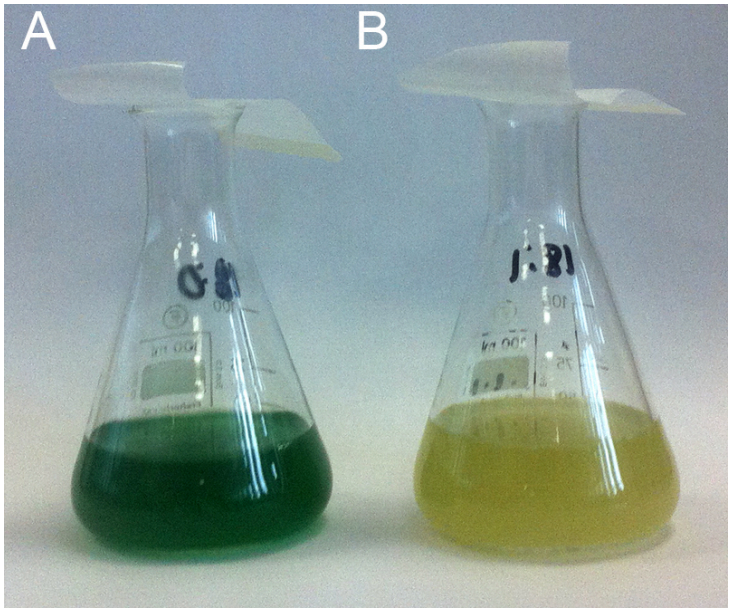


Figure 5.2. High concentrations of oleic acid is toxic to cyanobacteria. **A.** PCC 6803 after being fed for 24 h with 5 mM stearic acid in 1% ethanol under normal conditions. **B.** Dead PCC 6803 after being fed for 24 h with 5 mM oleic acid in 1% ethanol under normal conditions.

5.3 Oxygen electrode

5.3.1 Light Response Curves

An investigation into the light response of strains PCC 6803 and PCC 7120 was carried out using the oxygen electrode to give a light response curve; from this it is possible to determine respiration rate, light compensation and saturation points and optimal light intensities for photosynthesis.

Both strains have very similar light response patterns (Fig 5.3) a 2-sample t-test confirmed that overall there was no significant difference in rate of photosynthesis between the two cultures, $P > 0.05$. PCC 6803 has a light compensation intensity of just over 10 μ moles m^{-2} s^{-1} and PCC 7120 has a slightly higher intensity of 16 μ moles

m^{-2} s^{-1}. Light saturation point is reached at approximately 300 μ mol m^{-2} s^{-1} for both strains.

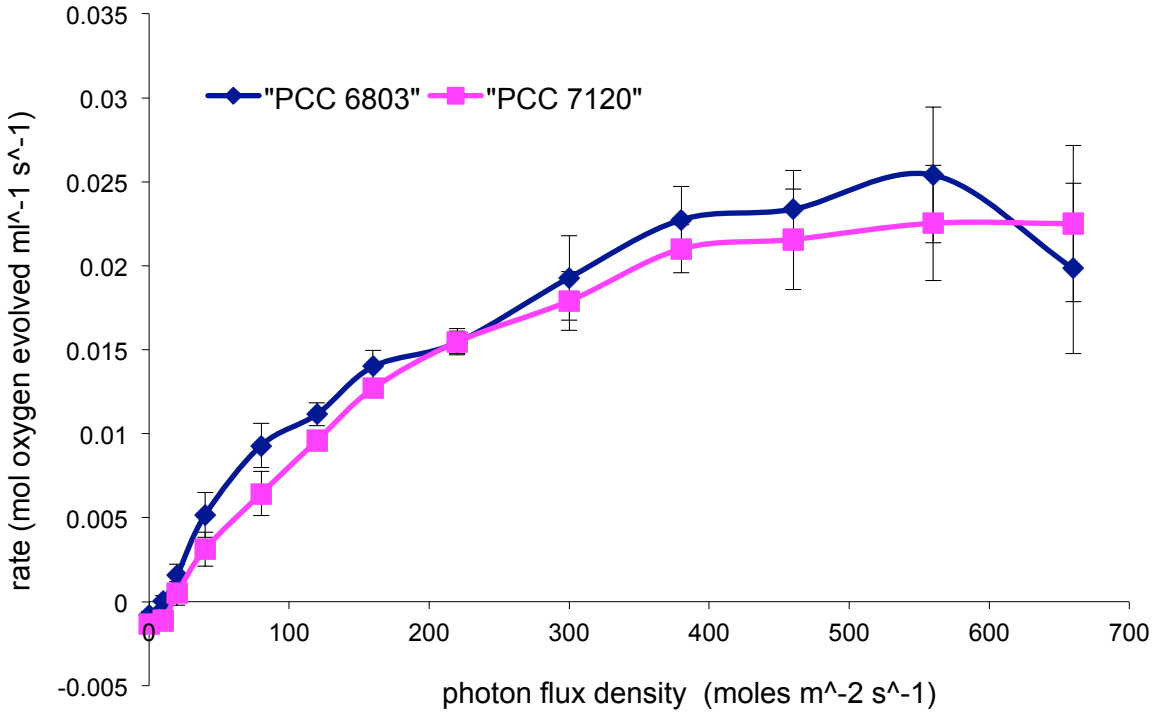


Figure 6.3. Light response curves of PCC 6803 and PCC 7120. ± S.E. n=3.

5.3.2 Feeding of fatty acids to cyanobacteria

A relatively simple initial experiment was conducted to help determine whether cyanobacterial cultures possess the β-oxidation pathway. This involved taking the respiration rate of cyanobacteria in the dark using the oxygen electrode, after 5 minutes a carbon source was spiked into the sample and respiration rate was taken again to determine if it had increased over the basal rate.

E. coli respired acetate at a rate around 10 times greater than cyanobacteria and could respire glucose at a rate around 20 times greater than that of PCC 6803 or 200 times greater than PCC 7120 (Fig. 5.4). Cyanobacterial cultures that are deprived of light generally were able to better utilize the exogenous carbon sources that were tested.

Light starved PCC 6803 could respire acetate, however PCC 7120 could not. Light starved PCC 6803 showed respiration of valeric acid, although in PCC 7120 addition of valeric acid was observed to completely halt respiration from the basal rate. PCC 6803 showed little respiration activity from C18 fatty acids, whereas PCC 7120 respires fatty acids of C10, C16 and C18 chain length. The only fatty acids tested that *E. coli* appeared to be able to respire were palmitate and oleate.

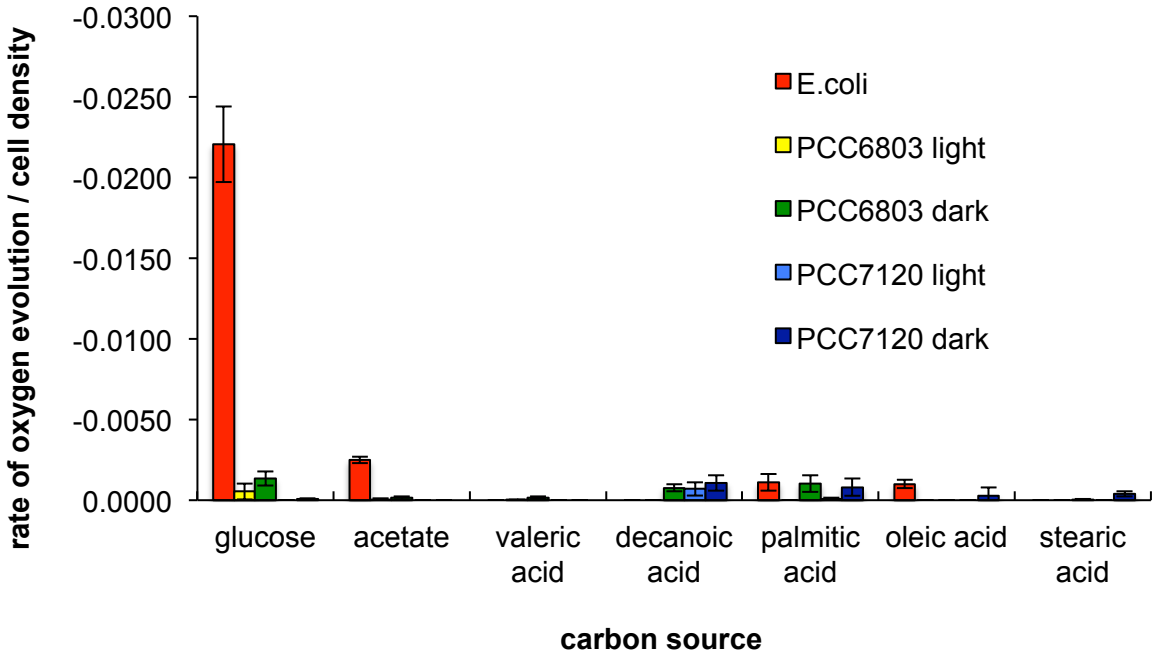


Figure 5.4. Respiration rates of *E. coli* (positive control), PCC 6803 and PCC 7120, under normal conditions and light starved conditions. Units of rate of oxygen evolution/cell density are in μmol ml^{-1} s^{-1} OD^{-1}. ±S.E. n=4.

5.4 Investigating the effect of feeding fatty acids on heptadecane yield of cyanobacteria using GCMS

By using GCMS it was possible to determine the presence of intracellular hydrocarbons in extracts of cyanobacteria. The main compounds detectable using this instrument were heptadecane, methyl-heptadecane, phytols, derivatised fatty acids and some photosynthetic accessory compounds in minor amounts.

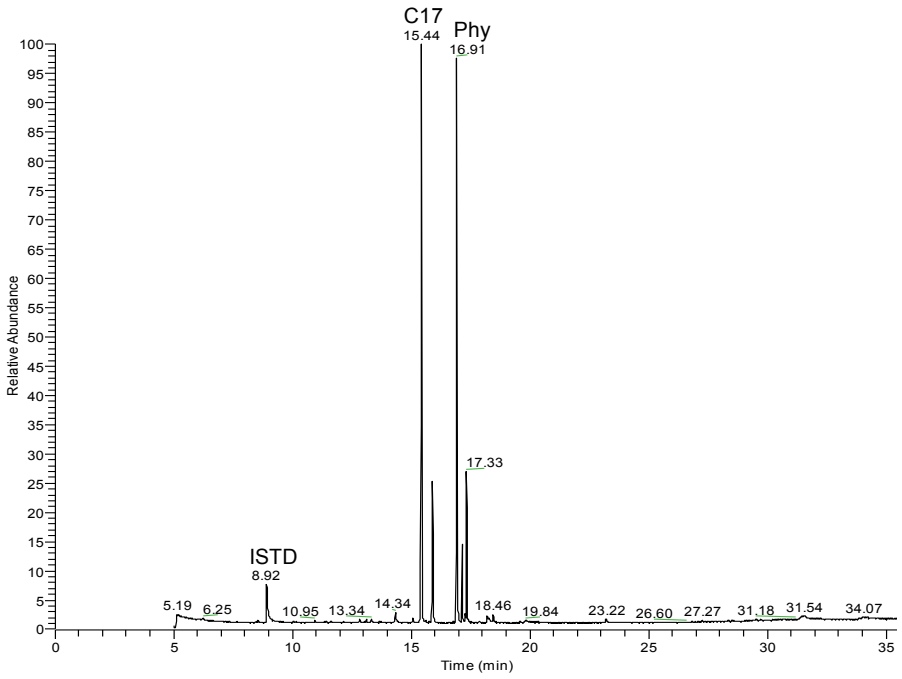


Figure 5.5. Example of a GCMS chromatogram. Internal standard (ISTD) D_8-napthalene elutes at 8.92 min; heptadecane (C17) elutes at 15.44 min, also methyl-heptadecane peak is observable in PCC 7120 and PCC 7937, eluting at 15.9 min; phytol (Phy) peaks elute between 16.91 min and 17.33 min.

A standard curve of heptadecane was constructed by injecting heptadecane standard solutions in DCM at 5, 10, 25 and 50 μg ml^{-1} concentrations, total heptadecane

measured in ng (Fig. 5.6), from this a y intercept value was calculated, and μg of heptadecane per mg of dry mass of cyanobacteria can be calculated using the equation:

$$Q = (A/I) / M$$

Where A is measured integrated peak area of heptadecane; I is intercept value, in this case 168397; and M is mass of cells, measured in mg.

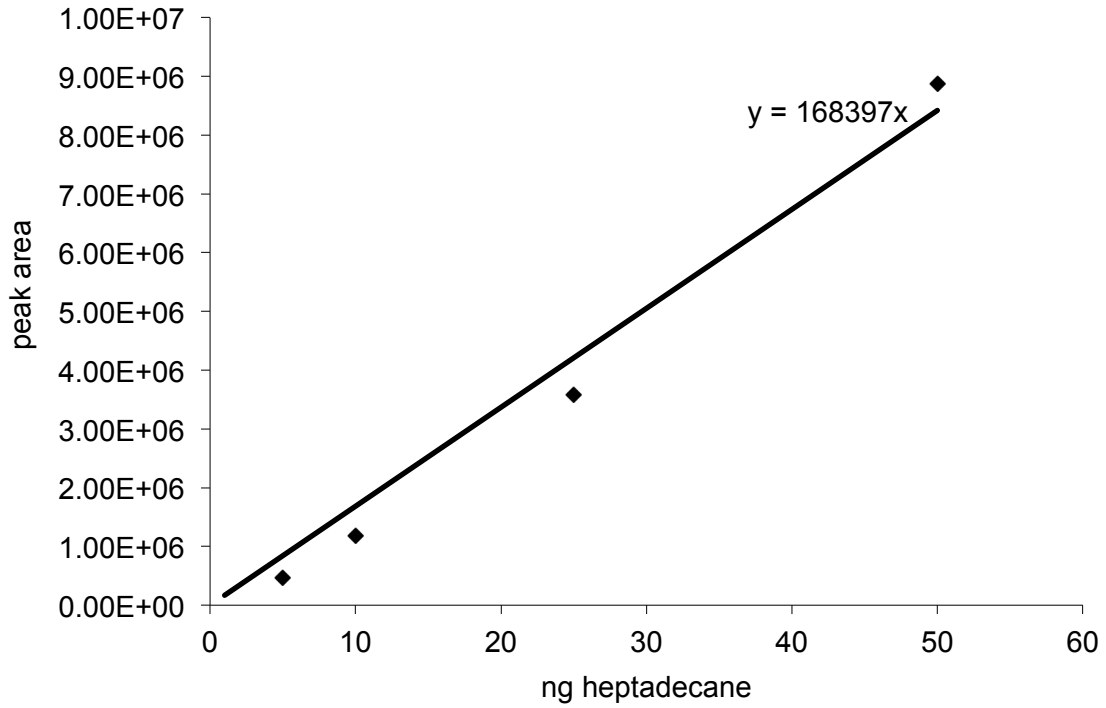


Figure 5.6 Heptadecane standard calibration curve. 5, 10, 25 and 50 μg ml^{-1} heptadecane (total heptadecane in ng) was injected into the GCMS instrument and resulting peak area recorded. y intercept value was calculated as 168,397.

Under normal conditions all three strains tested have similar concentrations of heptadecane (Fig. 5.8.), PCC 6803 having 1.89 ng mg^{-1}, PCC 7120 2.26 ng mg^{-1} and PCC 7937 1.56 ng mg^{-1}. When fed acetate, the yield of heptadecane in PCC 7120

increases by over 2.5 fold to 6.16 ng mg^{-1}, but in the other two strains this is not observed – with no substantial decrease in PCC 7937 and more than a 10-fold decrease in PCC 6803.

Feeding of fatty acids to PCC 6803 causes a 2 to 3 fold increase in heptadecane yield over the ethanol control. 18-carbon fatty acids, stearic acid and oleic acid cause heptadecane yield to fall by half compared to ethanol control in PCC 7120. No change in heptadecane yield over ethanol control are observed when PCC 7937 was fed palmitic acid or stearic acid, but a three fold increase observed when fed decanoic acid and oleic acid was observed.

When cultures were fed ethanol, the presence of long-chain ethyl esters was also detected at very high abundance (Fig. 6.7).

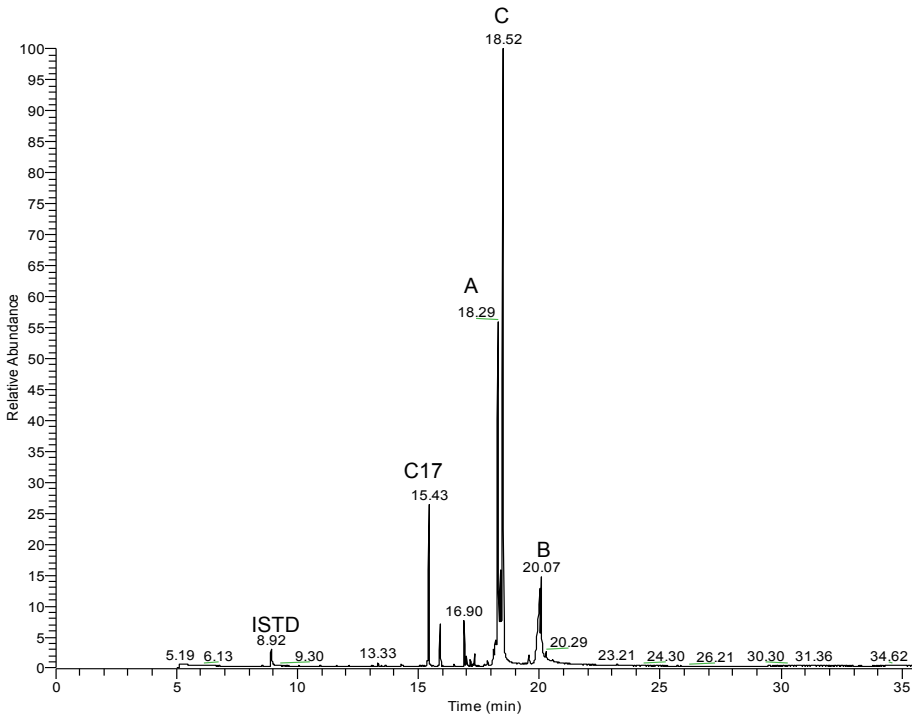


Figure 5.7. GCMS chromatogram of PCC 7120 when fed ethanol for 24 h. ISTD = D_8-napthalene internal standard; C17 = heptadecane; A = ethyl-9-palmitoleoylate; B = palmitic acid ethyl-ester; C = stearic acid ethyl ester.

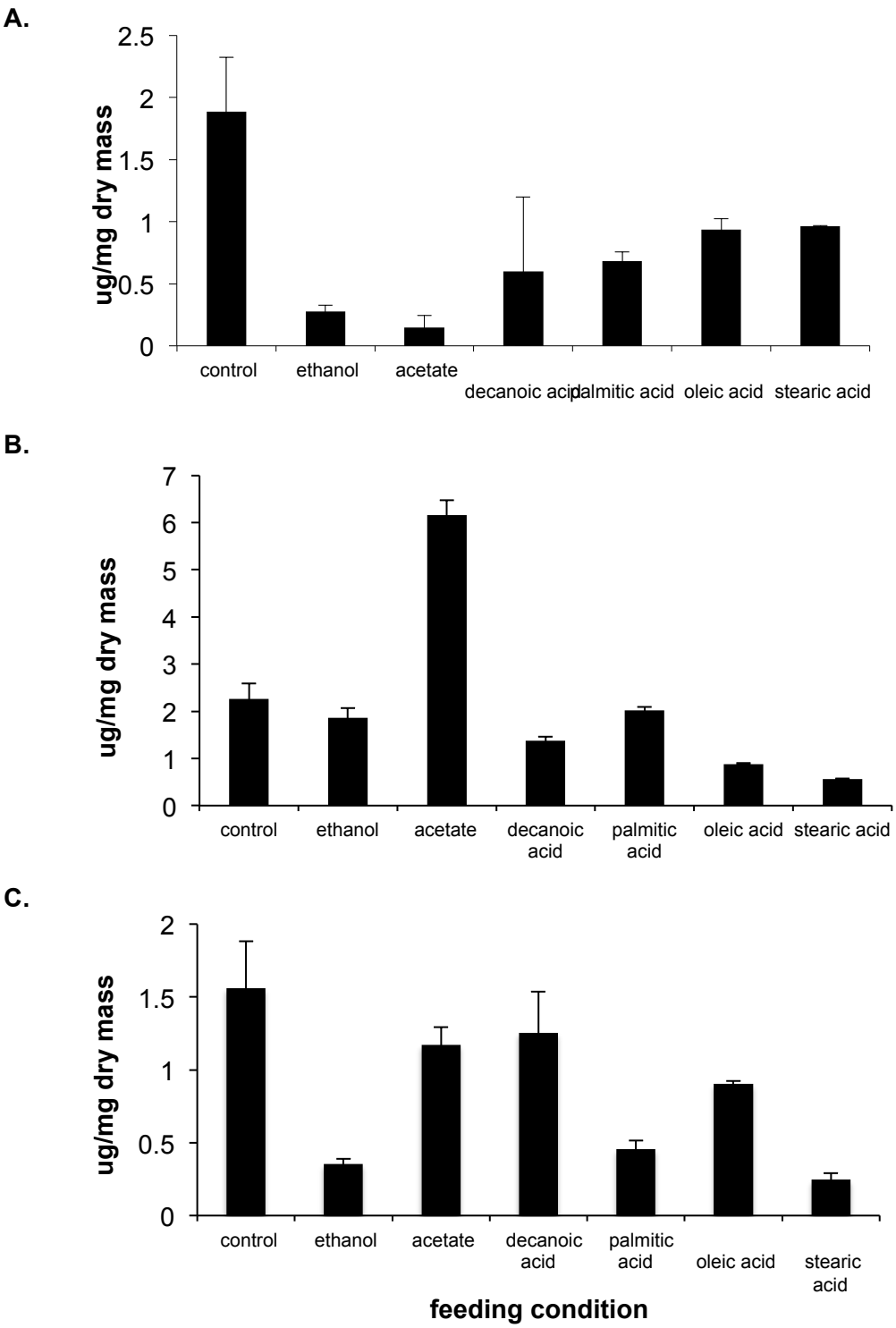


Figure 5.8. Heptadecane concentrations under a range of feeding conditions in PCC 6803 (**A**), PCC 7120 (**B**) and PCC 7937 (**C**) quantified as μg of heptadecane per mg of dry mass. Blank for acetate is control. Blank for fatty acids is ethanol. ± S.E. n=6.

5.5 Proteomic analysis of *Synechocystis* PCC 6803

An initial investigation on the proteome of PCC 6803 using nano-ESI-LC-QToF-MS was conducted to assess the feasibility of using this tool for later studies on fatty acid biosynthesis and degradative protein expression. From the protein samples prepared, 134 different proteins were detected over 3 runs. These proteins were classified according to their function (Fig. 5.9).

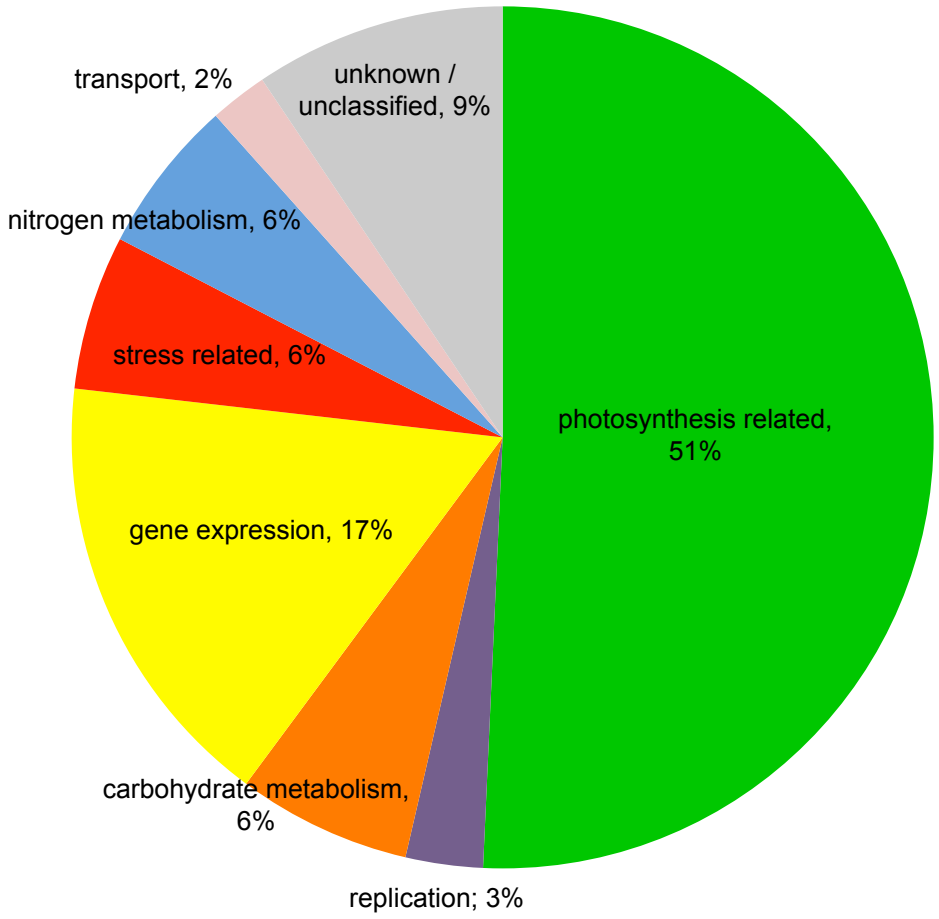


Figure 5.9. Protein classes based on function, detected in protein extracts of PCC 6803 detected over 3 runs using nano-ESI-LC-QToF-MS.

Of these proteins detected and identified using Spectrum Mill, none were related to fatty acid biosynthesis, degradation or regulation. Many proteins that are not of interest are present in such high abundances that they are effectively drowning out the signal from the proteins that are of interest for this project. An attempt was made to create a mass exclusion list using the data acquisition software in order to ignore any peptide fragments arising from these proteins that are not of interest. However, different fragments from the same protein were detected instead of the one excluded.

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